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Innate Immune Response to Coxiella Burnetii

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Abstract

Intracellular bacteria pose a unique set of challenges for the immune system. Many have evolved highly specialized virulence factors to evade intracellular detection and created a replicative niche. *Coxiella burnetii* is a zoonotic intracellular pathogen capable of causing severe acute and chronic Q fever in humans. *C. burnetii* grows in the lysosome and persists in cells for more than 10 days. Since *C. burnetii* can replicate inside cells for a prolonged period, we hypothesized that *C. burnetii* evolved multiple mechanisms to subvert innate immune detection. To test this hypothesis, we tested the ability of the *C. burnetii* Nine Mile Phase II clone 4/RSA439 strain to activate innate immune responses in restrictive C57BL/6 bone marrow-derived macrophages, specifically: detection by surface and cytosolic immune sensors; activation of the inflammasome; and production of Type I interferon. We found *C. burnetii* does not robustly stimulate cytosolic sensors, activate the inflammasome, nor induce robust levels of Type I interferon. These pathways do not play a significant role in mediating cell-intrinsic control of bacterial replication. We found TLR detection, specifically TLR2 and TLR4, primarily mediates innate immune detection and restriction of *C. burnetii* in C57BL/6 macrophages. We further implicated both TLR adaptors MYD88 and TRIF in this response. Building upon these findings, we demonstrated soluble TNF induced by TLR signaling plays a major role in the cell-intrinsic restriction of *C. burnetii*. We further examined TNF-dependent mechanisms of restriction: cell death, reactive nitrogen (RNS) and reactive oxygen (ROS) species, and guanylate binding proteins (GBPs). We found cell death is not induced in macrophages. Although expression of GBPs are induced upon *C. burnetii* infection, we ruled out five GBPs as mediators of bacterial restriction. Finally, we found RNS is induced by *C. burnetii* in a TNF-dependent manner, and ROS is also induced and plays a role in the cell-intrinsic control of *C. burnetii* replication. Together, these studies demonstrate that the *C. burnetii* Nine Mile phase II strain is able to evade many arms of the innate immune system in C57BL/6 macrophages and implicated a specific molecule, TNF, in mediating the cell-intrinsic control of this poorly understood bacterium.

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William P. Bradley

ABSTRACT

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Sunny Shin

Intracellular bacteria pose a unique set of challenges for the immune system. Many have evolved highly specialized virulence factors to evade intracellular detection and created a replicative niche. *Coxiella burnetii* is a zoonotic intracellular pathogen capable of causing severe acute and chronic Q fever in humans. *C. burnetii* grows in the lysosome and persists in cells for more than 10 days. Since *C. burnetii* can replicate inside cells for a prolonged period, we hypothesized that *C. burnetii* evolved multiple mechanisms to subvert innate immune detection. To test this hypothesis, we tested the ability of the *C. burnetii* Nine Mile Phase II clone 4/RSA439 strain to activate innate immune responses in restrictive C57BL/6 bone marrow-derived macrophages, specifically: detection by surface and cytosolic immune sensors; activation of the inflammasome; and production of Type I interferon. We found *C. burnetii* does not robustly stimulate cytosolic sensors, activate the inflammasome, nor induce robust levels of Type I interferon. These pathways do not play a significant role in mediating cell-intrinsic control of bacterial replication. We found TLR detection, specifically TLR2 and TLR4, primarily mediates innate immune detection and restriction of *C. burnetii* in C57BL/6 macrophages. We further implicated both TLR adaptors MYD88 and TRIF in this response. Building upon these findings, we demonstrated soluble TNF induced by TLR signaling plays a major role in the cell-intrinsic restriction of *C. burnetii*. We further examined TNF-dependent

mechanisms of restriction: cell death, reactive nitrogen (RNS) and reactive oxygen (ROS) species, and guanylate binding proteins (GBPs). We found cell death is not induced in macrophages. Although expression of GBPs are induced upon *C. burnetii* infection, we ruled out five GBPs as mediators of bacterial restriction. Finally, we found RNS is induced by *C. burnetii* in a TNF-dependent manner, and ROS is also induced and plays a role in the cell-intrinsic control of *C. burnetii* replication. Together, these studies demonstrate that the *C. burnetii* Nine Mile phase II strain is able to evade many arms of the innate immune system in C57BL/6 macrophages and implicated a specific molecule, TNF, in mediating the cell-intrinsic control of this poorly understood bacterium.

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CHAPTER 1:

Introduction: Immune Response to *Coxiella burnetii*

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General Introduction

Successful protection of hosts from pathogenic bacteria, viruses, parasites, and fungi involves the activation of complex immune networks that are also tightly regulated to prevent damage to the host. Multicellular organisms have evolved a diverse set of innate immune responses to prevent infection and establish long-lasting immunity. The successful clearance of a pathogen is first initiated by a series of sensor molecules that date back to single organisms and are present in all eukaryotes. These sensors, termed Pattern Recognition Receptors (PRRs), aid host organisms in distinguishing between pathogenic and non-pathogenic bacteria, while at the same time limiting the potential of recognizing self-associated molecules (Janeway and Medzhitov, 2002; Medzhitov, 2007; Vance et al., 2009). Intracellular bacteria pose a unique set of challenges for the immune system. Living inside the host cell shields them from recognition by complement, antibodies, and circulating immune cells. Many of these intracellular bacteria have evolved strategies to hijack host machinery to both prevent detection and establish a replicative niche.

Coxiella burnetii, the causative agent of Q (query) fever, is a poorly understood gram-positive intracellular bacteria. It is the only known bacteria to inhabit and replicate in the lysosome, an organelle often thought of to be the dead end of a bacteria's life cycle (Voth and Heinzen, 2007). Q fever can cause acute and chronic infections and is transmitted via an aerosol route with alveolar macrophages as the primary cell target (Raoult et al., 2005). Remarkably, as few as one bacterium can cause productive infection (Moos and Hackstadt, 1987). Once internalized by host cells, *C. burnetii* uses a specialized secretion system, termed the Type IV secretion system (T4SS), to inject effector proteins that are thought to target host cell machinery to aid in the maturation

and expansion of its unique parasitophorous vacuole (PV) (Heinzen et al., 1996; Seshadri et al., 2003; van Schaik et al., 2013). Inside the cell, *C. burnetii* is able to survive and replicate for days and form a vacuole that occupies the majority of the host cell cytosol (van Schaik et al., 2013). Systemically, *C. burnetii* does not induce robust neutrophil recruitment and requires an adaptive immune response for clearance (Elliott et al., 2013). In some instances, *C. burnetii* is able to overcome innate and adaptive immune barriers and establish chronic infection, spreading to the heart and liver to cause severe pathology in the host organism (Maurin and Raoult, 1999; Raoult et al., 2005). Despite its relatively early discovery in 1937, little is known about the innate immune response to *C. burnetii* (Derrick, 1983; Kováčová and Kazár, 2002). Due to the potential to cause severe disease in humans, its global presence (Marrie, 2010), and the potential to be used as a bioterrorism agent (Hackstadt, 1996), better understanding of the host immune response to *C. burnetii* and its evasion strategies are of high importance to limit the health and economic burdens of this disease.

Innate Immune Detection of Pathogens

Surface sensors

In host defense against pathogens, pattern recognition receptors that reside at the cell surface serve as the first line of detection to distinguish pathogenic and non-pathogenic bacteria. All eukaryotic organisms possess one or more of these receptors, which sense a broad array of pathogen-specific molecules. In vertebrate animals, surface and endosomal Toll-like receptors (TLRs) recognize molecular patterns, many of which are unique to bacteria, viruses, eukaryotic parasites, and fungi. Mice encode twelve functional TLRs, while humans encode ten. TLRs 1, 2, 4, 5, 6, 10 are exist on the cell

surface, while TLRs 3, 7, 8, and 9 are present in endosomes (Akira, 2003). These receptors use their N-terminal extracellular leucine-rich repeat domains to bind conserved microbial products, termed Pathogen-Associated Molecular Patterns (PAMPs) (Janeway and Medzhitov, 2002), or host stress signals, termed Danger-Associated Molecular Patterns (DAMPs). Upon recognition of foreign or host danger signals, TLRs undergo a conformational change in their C-terminal Toll/interleukin-1 receptor (TIR) domain. This conformational change activates cytosolic adaptor proteins which in turn activate signaling cascades to initiate anti-microbial responses (Akira, 2003). Monocytes, macrophages, and dendritic cells (DCs) constitutively express all TLRs while neutrophils, B cells, T cells and epithelial cells express only a few (Kumar et al., 2009). Each TLR has a distinct repertoire of ligands. The first characterized TLR, TLR4, was found to recognize lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall, but has been found to also recognize host heat shock protein, glycoproteins, and the fusion protein of respiratory syncytial virus (RSV) (Kawai and Akira, 2009). TLR2 recognizes both gram-negative and gram-positive bacterial-associated lipoproteins, peptidoglycan, and lipoteichoic acid (LTA), TLR2 can also dimerize with TLR1 or TLR6, providing an extra layer of ligand specificity. TLRs 3, 7, 8, and 9 all recognize foreign nucleic acids such as double-stranded RNA and DNA. Many TLR ligands, however, have yet to be discovered and are of great interest in both infectious and autoimmune diseases (Akira, 2003; Kumar et al., 2009).

All TLRs, except TLR3, signal through the adaptor MyD88, which in turn activates IRAK4, which then activates the NF- κ B pathway and the mitogen-activated protein kinases (MAPKs) p38 and JNK. Activation of these pathways results in the transcription and translation of several pro-inflammatory cytokines, including TNF, IL-6, IL-12, and the

pro-forms of IL-1 α and IL-1 β . The adaptor Trif is activated by TLR3 and TLR4 and results in the phosphorylation of interferon regulatory factors (IRFs). IRF3 and IRF7 induce the production of type 1 interferons (IFNs) IFN α and IFN β . TLR4 can also signal through Trif to activate the NF- κ B pathway and induce pro-inflammatory cytokine production (Akira, 2003).

The specific TLRs triggered dictate the downstream cell-extrinsic and cell-intrinsic responses. The ability of surface sensors to detect diverse sets of ligands and induce a range of signaling cascades provide cells with finely tuned outcomes to a broad range of pathogenic and host-derived stressors. TLR sensing has been implicated in the intracellular and systemic restriction of intracellular pathogens *M. tuberculosis* and *L. pneumophila* (Abel et al., 2002; Akamine et al., 2005; Reiling et al., 2002; Spörri et al., 2006; Zhang et al., 2013).

Cytosolic sensing

In addition to surface sensors, many cell types possess an additional line of defense in the form of cytosolic sensors. Since many TLR ligands are expressed by both pathogenic and non-pathogenic or commensal microbes, eukaryotic cells have evolved strategies to respond to a phenomenon unique to many pathogenic microbes – cytosolic access.

Most viruses access the cytosol during their natural replication cycle. Some pathogenic bacteria and parasites access the host cytosol either through the production of pore-forming toxins, such as Listeriolysin O expressed by the gram-positive bacterium *Listeria monocytogenes* (Hamon et al., 2012), or through the use of specialized secretion systems, such as the Type III secretion systems (T3SS) employed by *Salmonella*

enterica and *Yersinia* spp. and the Type IV secretion systems (T4SS) used by *Legionella pneumophila* and *Brucella abortis* (Cornelis, 2006; de Figueiredo et al., 2015; Pujol and Bliska, 2005; Qiu and Luo, 2013). These multiprotein structures act as molecular syringes and inject effector proteins into the host cytosol to hijack host machinery and establish a replicative niche (Roy and Mocarski, 2007). While cytosolic access is required for these pathogens to establish infection, host cells have co-evolved innate immune sensors to respond to this invasion (Franchi et al., 2009; Kawai and Akira, 2009).

Cytosolic sensors respond to a variety of PAMPs. Similar to TLRs, the immunological outcome depends on the specific ligand and sensor. Cytosolic sensors induce signal transduction cascades analogous to TLRs to produce pro-inflammatory cytokines and Type I interferons. Some cytosolic sensors are further capable of inducing formation of a multiprotein complex termed the inflammasome, which activates the host enzymes caspase-1 and caspase-11 and induces a form of highly inflammatory cell death termed pyroptosis (Franchi et al., 2009).

One class of cytosolic sensors, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), contain 16 members that are subdivided into five structural families: NLRAs contain an acid-activation domain; NLRBs contain a baculovirus inhibitor of apoptosis domain; NLRCs contain a CARD domain; NLRPs contain a pyrin domain; and NLRXs contain an unidentified domain. The best characterized NLR family members are the NLRC receptors, NOD1 and NOD2, and the inflammasome-inducing NLRP receptors (Franchi et al., 2009; Inohara et al., 2005).

Like TLRs, different cell types express different sets of NLRs. NOD1 is ubiquitously expressed by macrophages, human mononuclear cells, epithelial cells in the airway and

intestine, and dendritic cells, (Chamaillard et al., 2003; Inohara et al., 2005). NOD2 is expressed at higher levels in phagocytic cells and Paneth cells of the small intestine (Carvalho et al., 2015; Kobayashi et al., 2005). NOD1 and NOD2 both recognize bacterial peptidoglycan secreted by bacteria via outer membrane vesicles or through their specialized secretion systems. NOD1 has been implicated in the recognition of *Chlamydomphila pneumonia* (Shimada et al., 2009), *Legionella pneumophila* (Berrington et al., 2010; Frutuoso et al., 2010; Shin et al., 2008), *Klebsiella pneumonia* (Regueiro et al., 2011), and *Pseudomonas aeruginosa* (Travassos et al., 2005), while NOD2 responds to *Streptococcus pneumonia* (Opitz et al., 2004), *L. pneumophila* (Berrington et al., 2010; Frutuoso et al., 2010; Shin et al., 2008), *E. coli* (Theivanthiran et al., 2012), *C. pneumonia* (Shimada et al., 2009), and *M. tuberculosis* (Ferwerda et al., 2005).

A second set of cytosolic sensors, retinoic-acid-inducible protein 1 (RIG-I)-like sensors (RLRs) and other nucleic acid sensors, have been established in anti-viral responses, but have recently been shown to respond to bacterial nucleic acids to induce Type I interferon production (Kawai and Akira, 2009; Yoneyama and Fujita, 2007). Notable examples in the context of bacterial infections include: DNA-dependent activator of IFN-regulatory factors (DAI), which response to *S. pneumonia* (Parker et al., 2011); leucine-rich repeat (in FLII) interacting protein 1, LRRFIP1, which responds to *L. monocytogenes* (Yang et al., 2010); RIG-I, MDA5, and RNA Pol III, which respond to *L. pneumophila* (Chiu et al., 2009; Monroe et al., 2009); and the absent in melanoma 2 (AIM2) inflammasome, which responds to *F. tularensis* and *L. monocytogenes* (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010).

The combined action of surface and cytosolic sensors provides host organisms with a broad and rapid barrier to many pathogens. Some pathogens, however, have evolved

strategies to evade one or more of these barriers and as a result, establish productive infection.

Outcomes of Innate Immune Sensing

The specific outcome of surface and cytosolic detection depends on the pathogen and its ability to activate these sensors. The consequences of innate immune activation can be grouped into two categories: cell-extrinsic and cell-intrinsic. Cell-extrinsic responses rely on cytokine, chemokine, and interferon signaling from the infected cell to neighboring cells in order to mount a multicellular immune response. Cell-intrinsic responses can occur via signaling from the sensors themselves or via the action of a secondary molecule such as pro-inflammatory cytokines.

Cell Extrinsic Immune Responses

Innate Immune Cell Activation and Recruitment

Upon stimulation of surface and cytosolic sensors, signaling cascades are activated to produce and secrete pro-inflammatory cytokines (e.g. TNF, IL-6, IL-12, IL-1 α , and IL-1 β), chemokines, and Type I interferons (Akira, 2003; Kawai and Akira, 2009). Pro-inflammatory cytokines and chemokines can activate and/or recruit other innate and adaptive immune cells to the site of infection. Type I interferons activate a set of immune genes that were classically thought to primarily induce an anti-viral response. Recently, Type I interferons have been implicated in host responses to bacterial infection, but the mechanisms underlying how they restrict bacterial replication remain poorly understood (Coers et al., 2007; Manzanillo et al., 2012; Monroe et al., 2010).

Activation of Humoral Immunity

B cells provide another arm of the immune response and are activated by cytokines released by stimulated CD4+ T cells or by TLRs expressed on B cells themselves.

Studies have shown that antibodies (Abs) mediate protection against pathogens through direct bactericidal activity, complement activation, opsonization, cellular activation via Fc or complement receptors, and Ab-dependent cytotoxicity. Ab-mediated protection has been described in host defense against the intracellular bacteria *Mycobacterium tuberculosis* (Teitelbaum et al., 1998), *L. monocytogenes* (Edelson et al., 1999), and *Ehrlichia chaffeensis* (Li et al., 2001; Winslow et al., 2000).

In addition to direct immune action, specific Ab isotypes have been shown to modulate Th1 activation via Fc receptors (FcR). Interaction with these serotypes augments the T cell response by inducing rapid pathogen uptake, processing, and presentation of pathogen associated antigens. FcR-mediated antibody interactions were implicated in the T cell immunity against *Chlamydia trachomatis* (Moore et al., 2002).

Activation of T cell response

The mounting of a T cell response is also mediated by pro-inflammatory cytokines. The cytokine IL-12 stimulates a Th1 CD4+ T cell response, which results in the release of IFN γ and TNF. These cytokines released by T cells make infected cells more anti-microbial by increasing production of reactive oxygen (ROS) and reactive nitrogen (RNS) species inside the infected cell to promote pathogen killing.

Cell-intrinsic immune responses

Detection of pathogens by surface and cytosolic sensors can result in a variety of cell-intrinsic immune responses to limit intracellular infection. These responses can be activated by the sensors themselves or through their induction of pro-inflammatory cytokines.

Cell death

Programmed cell death is one cell intrinsic mechanism to limit bacterial infection. The death of infected cells eliminates the pathogens intracellular niche and, in some instances, releases pro-inflammatory molecules to alert neighboring cells. Three classes of programmed cell death have been described: apoptosis, pyroptosis, and necroptosis (Ashida et al., 2011; Fink and Cookson, 2005).

Apoptosis is a non-inflammatory cell death and has two forms: intrinsic apoptosis results when intracellular stressors disrupt the mitochondria membrane, whereas extrinsic apoptosis occurs via activation of caspase-8 by death receptors present on the cell surface. Both of these apoptotic cascades converge on caspase-3 which in turn activates a series of events resulting in DNA fragmentation, membrane blebbing, deconstruction of cellular components, and cell death (Elmore, 2007).

Necroptosis, or regulated necrosis, occurs as a back up death when the extrinsic, caspase-8 apoptotic pathway is blocked. This caspase-independent form of cell death occurs through the TNF receptor (TNFR) which signals to TNFR-associated death protein TRADD to activate RIP1 kinase, which recruits RIP3 kinase to form the necrosome. The formation of the necrosome leads to cell death and the release of cell components containing danger associated molecular patterns (DAMPs), such as

heatshock protein, ATP, and mitochondrial DNA, which can stimulate the same set of PRRs as PAMPs (Pasparakis and Vandenabeele, 2015; Vandenabeele et al., 2010).

Pyroptosis is a pro-inflammatory form of cell death that occurs via the activation of the inflammasome. Inflammasome activation is a two step process. The first signal occurs when TLRs and cytosolic sensors upregulate inflammsome machinery and the pro forms of IL-1 α and IL-1 β . A second signal, such as flagellin or extracellular ATP, binds certain cytosolic sensors to activate caspases-1 and -11. Caspase activation then initiates cell death and release of pro-inflammatory IL-1 family cytokines (Casson and Shin, 2013; Fontana and Vance, 2011; Franchi et al., 2012).

Reactive oxygen and reactive nitrogen species

Stimulation of surface and cytosolic sensors can also induce the production of reactive oxygen species (ROS) and reactive nitrogen (RNS) species. ROS is generated by the NADPH phagocytic oxidase (Phox), while RNS is generated by inducible nitric oxide synthase (iNOS) (Nathan and Shiloh, 2000). Both complexes are expressed in mononuclear (monocytes and macrophages) and polymorphonuclear (neutrophils) phagocytes. Neutrophils express more ROS than macrophages, and macrophages express more RNS than neutrophils (Nathan and Shiloh, 2000).

ROS is generated upon the formation of a multimeric protein complex that is pre-synthesized in phagocytes. Two membrane-bound proteins, p22^{phox} and gp91^{phox}, interact with the cytosolic proteins p40^{phox}, p47^{phox}, and p91^{phox}. Upon activation by phagocytosis, the cytosolic components translocate to the phagosome and assemble with flavocytochrome *b*₅₅₈ to form the functional NADPH oxidase complex (Bylund et al., 2010). Oxygen radicals are formed via electron transfer, which in turn spontaneously

generate hydrogen peroxide intermediates. Highly toxic hydroxyl radicals and hypochlorous acid are then generated via the Fenton reaction or by cellular myeloperoxidase (MPO) (Imlay and Linn, 1988; Imlay, 2008; Tlili et al., 2011). These molecules likely target bacterial DNA and can also act as signaling molecules to induce pro-inflammatory cytokines and cell death.

TNF, IFN γ , and TLR ligands, such as LPS, stimulate the *de novo* synthesis of iNOS, which contains an N-terminal oxygenase and a C-terminal reductase (Vila-del Sol et al., 2007). iNOS forms homodimers and localizes to the phagosome in an actin dependent manner. It produces nitric oxide radicals from the L-arginine substrate (Albakri and Stuehr, 1996; Baek et al., 1993; Davis et al., 2007). Nitrogen radicals target a complex repertoire of bacterial components including bacterial DNA, proteins, and lipids. Bacterial death likely occurs through the disruption of bacterial respiration components (Nathan and Shiloh, 2000).

Intracellular pathogens have evolved strategies to evade RNS and ROS toxicity. To scavenge ROS, some pathogenic bacteria encode superoxide dismutases (SODs), catalases, and peroxiredoxins (Prxns) (Chelikani et al., 2004; Gort and Imlay, 1998; Parsonage et al., 2005). Low molecular weight thiols and free or residual methionine are utilized to scavenge both ROS and RNS (Boschi-Muller et al., 2005; Carmel-Harel and Storz, 2000).

Autophagy

Autophagy, specifically the subclass termed xenophagy, has been implicated in anti-bacterial defense. When endosomal trafficking of microbes to terminal lysosomes is subverted, autophagy provides a back up mechanism to degrade foreign invaders. In

phagocytes, recognition of PAMPs by both surface and cytosolic sensors to induce a microbicidal form of autophagy known as xenophagy (Levine et al., 2011).

Guanylate Binding Proteins (GBPs)

Recently a family of IFN γ -inducible GTPases termed guanylate binding proteins (GBPs) have been implicated in the cell-intrinsic control of intracellular pathogens. Humans encode seven GBPs (hGBP1-7), while mice encode eleven (mGBP1-11) (Olszewski et al., 2006; Vestal and Jeyaratnam, 2011). GBPs are classically thought to be induced by Type I and Type II interferons, but select GBPs have been shown to be induced by IL-1 α , IL-1 β , and TNF (Vestal and Jeyaratnam, 2011). Upregulated GBPs are targeted to cellular membranes by isoprenylation, or the addition of a C-15 farnesyl or C-20 geranylgeranyl lipid moieties to the cysteine in a conserved C-terminal CaaX sequence (Vestal and Jeyaratnam, 2011). Both mice and human GBPs have been implicated in the control of intracellular bacteria. hGBP-1 and hGBP-2 inhibit replication of *Chlamydia spp* (Tietzel et al., 2009); mGBP-5 promotes and mGBPs encoded on chromosome 3 have been implicated in *Salmonella Typhimurium* and *L. pneumophila* induced pyroptosis, respectively (Meunier et al., 2014; Pilla et al., 2014).

Bacterial Subversion of Sensing

To overcome the wide array of innate immune barriers, pathogenic bacteria have evolved many strategies to evade or subvert innate immune detection in order to establish infection. These strategies can be grouped into two categories: the alteration or deletion of PAMPs, and active inhibition of innate immune signaling via secreted bacterial effectors or toxins (Bhavsar et al., 2007).

Immune evasion by molecular changes via evolution

Some pathogens have altered their PAMPs to be non-stimulatory or antagonistic. One such variation occurs in the Lipid A portion of the LPS expressed by gram-negative bacteria. TLR4 preferentially recognizes hexa-acylated Lipid A with fatty acid side chains between 12-16 carbons in length (Miller et al., 2005; Roy and Mocarski, 2007). Species of *Legionella* and *Rhizobium* have increased the length of their fatty acid side chains to >20 carbons and as a result, are less stimulatory to TLR4 (Schroemm et al., 2000). *Helicobacter pylori*, a human pathogen of the stomach, encodes a tetra-acylated Lipid A with fatty acid chains ranging from 16-18 carbons, which is not sensed by TLR4 (Moran et al., 1997).

To avoid detection by flagellin sensors, such as TLR5 and NAIP5, some bacteria encode variations in their flagellin or downregulate its expression. Several members of the α - and ϵ - classes of the phylum *Proteobacteria* phylum, including *H. pylori*, encode mutations in the N-terminus of flagellin, rendering the molecule incapable of activating TLR5 (Gewirtz et al., 2004). Other bacteria that invade the mucosal epithelium, such as *Salmonella enterica* serovar Typhimurium and *Campylobacter jejuni*, regulate expression of their flagellin by turning off expression of the stimulatory molecule to successfully colonize the host (Kinsella et al., 1997; Simon and Samuel, 2007).

Gram-positive bacteria have also evolved strategies to evade detection. To shield their cell wall from TLR2 detection, *Streptococcus pneumoniae* and *Klebsiella pneumoniae* form a polysaccharide capsule (Benghezal et al., 2007; Geno et al., 2015; Regueiro et al., 2011). Distinct structural adaptations in both gram-positive and gram-

negative bacteria have allowed many species to thwart host recognition and establish productive infection.

Use of toxins or effector proteins to evade innate immunity

Bacteria also encode virulence factors that target and dampen innate immune responses. The gram-positive bacterium *Bacillus anthracis* encodes a metalloprotease toxin that targets and inactivates MAPKs, resulting in apoptosis of macrophages and dendritic cells. The induction of apoptosis is thought to prevent signaling from these immune cells to other cells, allowing *B. anthracis* to colonize the host (Collier and Young, 2003).

Some species of gram-negative bacteria encode specialized secretion systems, such as the Type III and Type IV secretion systems. These multi-protein complexes act as molecular syringes that are thought to form pores in cell surface or endosomal membranes and translocate effector proteins that target host machinery (Diepold and Armitage, 2015; Trokter et al., 2014). Many of these effector proteins have eukaryotic-like domains – likely the result of horizontal gene transfer. Effector proteins target numerous host processes, including various signaling cascades, vesicular transport, gene expression, and cell death pathways. Type III secretion systems are encoded by many bacteria, including *Yersinia spp.* and *Salmonella spp.*, and Type IV secretion systems are encoded by several other bacteria, including *Legionella spp.*, *Brucella abortis*, and *C. burnetii* (Diepold and Armitage, 2015; Trokter et al., 2014).

Coxiella burnetii

Epidemiology

Coxiella burnetii is a gram-negative, intracellular bacterium that causes Q (query) fever in humans. Q fever can manifest as an acute infection with flu-like symptoms or progress to a system chronic infection which can lead to endocarditis or hepatitis (Maurin and Raoult, 1999; Raoult et al., 2005). Livestock is the primary reservoir for *C. burnetii*, with many outbreaks linked to goats (Raoult et al., 2005; Whelan et al., 2011). The bacteria can persist in herds and spread in massive quantities during the livestock birthing process, and infection of pregnant livestock often results in stillbirths (Di Domenico et al., 2014; Raoult et al., 2005). Transmission leads to persistence within herds and puts nearby humans at a high risk of exposure. While human-to-human transmission is extremely rare, a recent three year outbreak in the Netherlands linked to dairy goat farms that resulted in over 4,000 human cases and mass culling of goat herds highlighted the potential health and economic burden *C. burnetii* can cause (Enserink, 2010; Roest et al., 2011). The only *C. burnetii* vaccine approved for human use is available solely in Australia. While proven effective at protecting non-exposed individuals, it can also cause severe adverse reactions in individuals previously exposed to *C. burnetii* (Chiu and Durrheim, 2007). The outbreak potential, limited prophylaxis options, and potential use as a bioterrorism agent highlight the need for a better understanding of *C. burnetii* pathogenesis and how the host immune response can successfully control this organism.

Despite the relatively early discovery of *C. burnetii* in 1937, knowledge of other bacterial pathogens have far outpaced *C. burnetii* due to its BSL-3 and Select Agent designation and history as an obligate intracellular pathogen, with past difficulties with cell-free growth (Derrick, 1983; Raoult et al., 2005). The generation of an avirulent BSL-2 lab strain and the recent development of axenic media has opened doors for a better

molecular understanding of *C. burnetii*'s pathogenesis (Omsland et al., 2009; Vishwanath and Hackstadt, 1988).

The primary entry route for *C. burnetii* in humans is through inhalation into the lung and infection of alveolar macrophages (Raoult et al., 2005; van Schaik et al., 2013). While it is known that *C. burnetii* can infect any nucleated cell, it is thought to prefer macrophages (Elliott et al., 2013). The incubation time prior to the onset of disease symptoms varies from a few days to months, a relatively long period compared to other pathogens (Raoult et al., 2005). Acute infection occurs in ~40% of exposed individuals, where patients experience flu-like symptoms that can last for days to weeks (Harris et al., 2000). Cases of acute infection are largely self-limiting and can be treated with a two week regiment of doxycycline (Raoult et al., 2005). 1-5% of acute presentations can progress to a more severe chronic infection which can have devastating consequences (Tissot-Dupont and Raoult, 2008). In chronically infected individuals, *C. burnetii* spreads to the heart and liver and can cause severe endocarditis or hepatitis, with patients often needing heart valve replacement surgery or liver transplants (Tissot-Dupont and Raoult, 2008).

Coxiella burnetii biology

C. burnetii is a gram-negative intracellular bacteria of the phylum *Proteobacteria*, class γ -*Proteobacteria*, order *Legionales*, class *Coxiellaceae* (Maurin and Raoult, 1999; Roux et al., 1997; Stein et al., 1993). *C. burnetii*'s genome contains ~2.0-2.2 Mb with ~57.8% AT's, which is thought to aid in homologous gene transfer of eukaryotic-like proteins that aid in its pathogenesis (Raghavan et al., 2008; Seshadri et al., 2003). It is a relatively small bacteria with its size ranging from 0.2-0.4 μ M wide and 0.4-1 μ M long,

and it is highly resistant to environmental stressors such as dessication and heat (Raoult et al., 2005). Its replication time is estimated at 12-20 hours (Zamboni et al., 2002).

C. burnetii has a pleomorphic coccobacilli shape and exists as two morphologically distinct cell types. The environmentally stable, non-replicative, small cell variant (SCV) is characterized by condensed chromatin and is thought to be the more infectious of the two variants (Hackstadt and Williams, 1981). The replicative, large cell variant (LCV) develops upon internalization by the host cell or when introduced into nutrient-rich, acidified citrate cysteine medium (ACCM) (Coleman et al., 2004; Hackstadt and Williams, 1981; Omsland et al., 2013). This morphological shift is marked by alterations in the surface protein composition, activation of metabolism pathways, and replication (Coleman et al., 2004; 2007). Once the bacteria reaches stationary phase either inside the host cell or in axenic media, the bacteria revert back to the small cell variant (Coleman et al., 2004; 2007; Omsland et al., 2013).

Further variations occur with the bacterium's lipopolysaccharide. The smooth, virulent form (Phase I) contains a highly branched O-chain, containing the unusual sugars virenose (6-deoxy-3-C-methyl-D-gulose) and dihydrohydroxystreptose (3-C-(hydroxymethyl)-L-lyxose (Amano et al., 1987; Moos and Hackstadt, 1987; Toman and Kazár, 1991). This branching is thought to aid in the evasion of serum-mediated killing (Vishwanath and Hackstadt, 1988). The attenuated and laboratory adapted Nine Mile Phase II clone 4/RSA439 strain is the standard lab strain, and was derived from the virulent Nine Mile phase I strain via serial lab passages in embryonated eggs. Serial passage resulted in the deletion of large chromosomal regions containing open reading frames associated with O-chain synthesis, which is thought to account for its attenuated virulence *in vivo* (Moos and Hackstadt, 1987).

Coxiella burnetii inside the host cell

C. burnetii is internalized by two mechanisms: active phagocytosis and passive binding to leukocyte response integrin ($\alpha_v\beta_3$ integrin) and CR3 receptor (Boyle and Finlay, 2003; Capo et al., 1999; Dellacasagrande et al., 2000). Once internalized, *C. burnetii* traffics down the canonical endosomal pathway, terminating in an acidic, lysosome-like compartment (Heinzen et al., 1996; Kinchen and Ravichandran, 2008). The *C. burnetii*-containing vacuole also engages autophagy pathways, and can undergo homotypic fusion with other *C. burnetii*-containing vacuoles (Heinzen et al., 1996; Romano et al., 2007). Autophagic engagement is thought to provide the bacteria with nutrients and membrane for vacuole expansion (Romano et al., 2007). Unlike *Legionella*, which blocks lysosomal maturation of its vacuole (Isberg et al., 2008), *C. burnetii* traffics to a fully mature phagolysosome containing both early endosomal marker Rab5 and late endosomal marker Rab7 (Berón et al., 2002). The mature PV also contains the lysosome-associated marker LAMP-1, the lysosomal hydrolase acid phosphatase, and cathepsin D (Heinzen et al., 1996; Howe et al., 2010). Upon lysosomal maturation, *C. burnetii* begins to form its intracellular niche, the parasitophorous vacuole, which takes ~24-48 hours to mature (Heinzen et al., 1996; Howe et al., 2003). Approximately 8 hours post internalization, *C. burnetii* undergoes a morphological switch from the infective small cell variant to replicative large cell variant and begins injecting effector proteins into the host cell via its Type IV secretion system (Newton et al., 2013). These effectors are thought to hijack host cell machinery to enable the acquisition of nutrients and host membrane, and prevent host cell death (Coleman et al., 2004; Heinzen et al., 1996; van Schaik et al., 2013). *C. burnetii* continues to replicate in this PV for days, forming a large

vacuole that can occupy the majority of the host cell cytosol and can contain upwards of 100 bacteria per vacuole (Coleman et al., 2004).

Coxiella burnetii's Type IV Secretion System

C. burnetii encodes a Type IV secretion system (T4SS) that delivers bacterial effector proteins to the host cytosol (van Schaik et al., 2013). *C. burnetii's* T4SS is highly homologous to *L. pneumophila's* Dot/Icm (defect in organelle trafficking/intracellular multiplication) T4SS system (Seshadri et al., 2003). Previous studies that identified many of the known *C. burnetii* T4SS effector proteins utilized *L. pneumophila* as a surrogate for translocation assays to screen or test potential *C. burnetii* effectors, as its T4SS is both genetically and functionally homologous to *L. pneumophila's* T4SS (Vogel, 2004; Zamboni et al., 2003). To date, over 100 effector proteins have been identified. Many of these effectors have eukaryotic domains and are targeted to distinct regions of the cell (van Schaik et al., 2013). Since a functional T4SS is necessary for bacterial replication (Beare et al., 2011), it is thought that these effector proteins hijack host machinery to aid in vacuole formation, nutrient acquisition, alteration of gene expression, and subversion of host immune responses. So far, three effectors, AnkG, CaeA, and CaeB, have been linked to the inhibition of intrinsic apoptosis (Klingenberg et al., 2013; Lührmann et al., 2010), and several effectors have been linked to vacuole formation (Graham et al., 2015; Larson et al., 2013; 2015; Newton et al., 2014; Weber et al., 2013).

Host recognition of Coxiella burnetii

Unlike *L. pneumophila*, which evolved to infect single cell amoeba and has not acquired virulence factors that specifically interfere with innate or adaptive immunity,

successful clearance of *C. burnetii* requires both innate and adaptive immune responses. The activation of innate immune pathways and the downstream adaptive response remain poorly understood.

TLR and NLR Sensing

Studies on innate immune recognition of *C. burnetii* have shown differing results depending on the bacterial strain or host cell used and have largely focused on TLR2 and TLR4. One study showed that Phase I *C. burnetii* productively replicates in human dendritic cells without stimulating TLR2 or the maturation of the DCs (Shannon et al., 2005). Another study demonstrated that *C. burnetii* Phase II does stimulate TLR2, resulting in the secretion of TNF (Zamboni, 2004). In this study, TLR2 was found to play a role in inhibiting growth in C57BL/6-background murine bone marrow-derived macrophages as *Tlr2*^{-/-} macrophages showed increased vacuole size and bacterial replication (Zamboni et al., 2003). A recent study further demonstrated that TLR2 signaling relied on heterodimerization with TLR1, while TLR6 deficiency did not result in an attenuated immune response in response to infection with the Nine Mile strain (Ammerdorffer et al., 2014). It is hypothesized that the highly branched Phase I LPS acts as a shield and prevents exposure of TLR2 agonists, which could explain the previously described robust Th1 response in mice infected with Phase II, but not Phase I bacteria (Roy and Mocarski, 2007; Schromm et al., 2000).

The role of TLR4 is still unclear. It was found that TLR4 mediates bacterial uptake of Phase I, but not Phase II bacteria (Honstetter et al., 2004). Furthermore, the previous study linking TLR2 detection of *C. burnetii* to production of TNF in mouse macrophages demonstrated purified *C. burnetii* lipid A was a TLR4 antagonist (Zamboni et al., 2004).

The same study implicating TLR1-mediated detection of *C. burnetii* also showed that NOD2 plays a role in cytokine production, providing insight into the first potential cytosolic sensor activated by *C. burnetii* (Ammerdorffer et al., 2014).

Neutrophil Recruitment

Neutrophil recruitment to the site of infection is a major defense mechanism against a variety of lung pathogens, usually occurring around 24 to 48 hours post-infection (Balamayooran et al., 2010). It was shown that *C. burnetii* delays neutrophil recruitment for approximately seven days (Elliott et al., 2013). The same research group further performed a study indicating that neutrophils are important for clearance, as antibody-mediated depletion of neutrophils resulted in more severe infection (Elliott et al., 2013). The recruitment of neutrophils during *C. burnetii* infection was found to be independent of both CXCL2 and IL-17, two classical neutrophil recruitment molecules (Balamayooran et al., 2010; Elliott et al., 2015).

Adaptive Immune Response

Successful clearance of *C. burnetii* requires an adequate adaptive immune response. Studies in SCID and nude mice, which lack both T and B cells, are highly susceptible to *C. burnetii* infection (Andoh et al., 2003; Kishimoto et al., 1978). Furthermore, immunocompromised individuals, such as patients on immunosuppressive treatment, with lymphomas, or pregnant individuals are more likely to develop chronic Q fever (Capo and Mege, 2012).

Successful control of acute Q fever relies on a systemic T cell response, likely Th1, and granuloma formation (Maurin and Raoult, 1999). SCID mice reconstituted with CD4⁺

T cells or CD8⁺ T cells are able to control infection (Read et al., 2010). The T cell response is thought to rely heavily on the production of IFN γ , as IFN γ -deficient mice exhibit high mortality (Andoh et al., 2007). The exact microbicidal programs that IFN γ stimulates to control *C. burnetii* infection remain unclear. T cells are also involved in the formation of granulomas, which are thought to be necessary for clearance during acute phase because patients with chronic Q fever have fewer granulomas (Koster et al., 1985a; 1985b).

It is known that both infection with *C. burnetii* and injection of *C. burnetii* antigens into mice induces a significant antibody response in animals and humans (Behymer et al., 1975; Guigno et al., 1992; Kishimoto and Burger, 1977; Peacock et al., 1979). Patients with acute Q fever have been shown to develop IgM specific antibodies to Phase I antigen and IgM, IgG, and IgA antibodies to Phase II antigen, whereas patients with chronic Q fever did not produce detectable levels of IgM, but had high levels of both IgA and IgG (Worswick and Marmion, 1985). Mixtures of virulent *C. burnetii* and antibody were found to be noninfectious when injected into animals, and studies with formalin-killed *C. burnetii* vaccines in humans directly linked antibodies to the control of *C. burnetii* infection (Lackman et al., 1962; Ormsbee et al., 1964; Peacock et al., 1979). Administered *C. burnetii* antibodies were unable, however, to control infection in T cell-deficient animals, indicating that T cells are necessary for antibody-mediated clearance (Andoh et al., 2007; Humphres and Hinrichs, 1981). It is currently hypothesized that a successful humoral immune response is necessary to control infection, while a successful T cell response is necessary to completely clear *C. burnetii* from the host.

Conclusions

Host cells have evolved a variety of mechanisms to recognize and control intracellular pathogens. All of these responses rely on the initial detection of the pathogen by innate immune sensors that reside at the cell surface or within the host cell cytosol. *Coxiella burnetii* is a poorly understood pathogen that has the potential to cause severe disease in humans. While many studies have been done to dissect role individual components play in the innate and adaptive control of *C. burnetii*, different findings have been arrived at using cells from different animal origins, immortalized, and primary cells. Here we sought to survey the innate immune response mounted by macrophages against the Nine Mile Phase II clone 4/RSA439 strain in a well-established system employed in the study of many pathogens, macrophages derived from C57BL/6 mice. Since wild-type C57BL/6 macrophages are restrictive to *C. burnetii* growth, we were able to use C57BL/6 background mice genetically deficient in key components of the host surface and cytosolic surveillance system to examine *C. burnetii*'s ability to activate canonical arms of the innate immune response and decipher the mechanisms underlying cell-intrinsic control of *C. burnetii* within C57BL/6 macrophages.

Dissertation Aims

Intracellular bacteria pose unique challenges to the host immune response. They have evolved to live and thrive in specialized intracellular niches, shielded from circulating immune challenges in the extracellular space such as antibodies and complement. Despite the presence of a diverse set of innate immune sensors on the host cell surface and in the cytosol that can detect microbial pathogens and activate a wide array of immune responses, pathogens have evolved strategies to subvert this detection and establish infection.

One intracellular bacterium, *Coxiella burnetii*, is a poorly understood pathogen capable of causing severe acute and chronic infection in humans. It is the only known bacterium to live and replicate in the harsh environment of the lysosome. It also is able to survive inside cells over a long period of time and delays recruitment of neutrophils by several days. **We hypothesize that *C. burnetii* has evolved strategies to evade or delay activation of innate immune pathways in macrophages by either failing to activate these pathways or blocking activation through the use of its specialized Type IV secretion system (T4SS).** While cells from many organisms are consusive to productive *C. burnetii* infection, macrophages from C57BL/6 (B6) mice are able to control *C. burnetii* replication via an unknown mechanism. **We, therefore, further hypothesize that despite a lack of robust innate immune activation, *C. burnetii* activates a cell-intrinsic innate immune pathway in B6 macrophages.** To address these overarching hypotheses, we pursued the following specific aims:

Aim 1: Determine which innate immune pathways are activated in mouse macrophages following *in vitro* infection with *C. burnetii*. In the first aim, we

investigated the ability of the *C. burnetii* Nine Mile Phase II clone 4/RSA439 strain to activate various components of the innate immune system in C57BL/6 bone marrow-derived macrophages. We specifically looked at: the role of surface TLRs and cytosolic sensors in production of proinflammatory cytokines, TNF and IL-6; the activation of the inflammasome; and the induction of Type I interferons. C57BL/6 naturally restrict *C. burnetii* so we further wanted to determine if these pathways play a role in cell intrinsic restriction. To expand upon our hypothesis that *C. burnetii* evolved strategies to evade innate immune detection, we further tested the role of its type IV secretion system in the activation of the innate immune response

Aim 2: Determine the mechanism(s) by which TLR-mediated production of TNF restricts *C. burnetii* replication within macrophages. We uncovered TNF as a major player in mediating cell-intrinsic restriction of *C. burnetii*. Since TNF induces a wide array of cell-intrinsic immune pathways, we hypothesized that at least one of these pathways acts to restrict *C. burnetii* growth. To test this hypothesis, we focused on three possible TNF-mediated restriction mechanisms: activation of cell death via apoptosis and necroptosis; the production of reactive nitrogen (RNS) and reactive oxygen (ROS) species; and the induction of guanylate binding proteins (GBPs). With each of these pathways, we determined if *C. burnetii* activated these pathways in C57BL/6 macrophages and then sought to determine if their activation was dependent on TNF and if they played a role in cell-intrinsic restriction of *C. burnetii*.

Together, these aims provide new insight into our understanding of the innate immune response to *C. burnetii* in macrophages (Aim 1) and implicated a specific host immune pathway in directing cell-mediated control of *C. burnetii* replication (Aim 2).

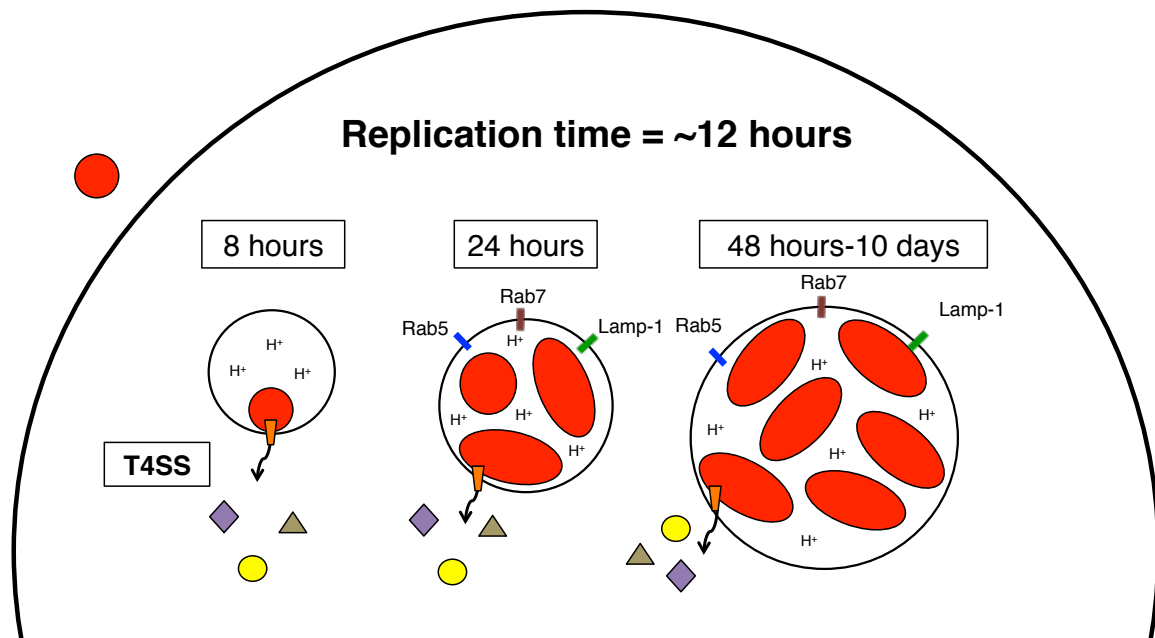


Figure 1.1 The intracellular lifestyle of *C. burnetii*.

C. burnetii is internalized by active phagocytosis or passive binding to leukocyte response integrin ($\alpha_v\beta_3$ integrin) and CR3 receptor. Internalized *C. burnetii* traffics down the canonical endosomal pathway, terminating in an acidic, lysosome-like compartment containing Rab5, Rab7, and LAMP-1. 8 hours post internalization, *C. burnetii* injects effector proteins into the host cell cytosol to form its replicative niche, the *Coxiella* containing vacuole (CCV). 24 hours post infection *C. burnetii* undergoes a morphological shift from the infective, metabolically inactive small cell variant (SCV) to the replicative large cell variant (LCV). Once the CCV is fully formed, the bacteria replicate inside the lysosome-like vacuole at doubling time of ~12 hours. This replication period persists for ~10 days, with the CCV occupying the majority of the host cell cytosol. At stationary phase, *C. burnetii* reverts back to its infective SSV and exits the cell via an unknown mechanism.

Chapter 2:
Innate immune recognition and restriction of
Coxiella burnetii in C57BL/6 macrophages

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Abstract

Detection of intracellular bacterial pathogens by extracellular and cytosolic pattern recognition receptors is a critical first step in mediating cell-intrinsic restriction of bacterial replication. The intracellular pathogen *Coxiella burnetii* replicates within permissive cell types by using a Dot/Icm type IV secretion system (T4SS) to translocate bacterial effectors into the host cell cytosol. C57BL/6 (B6) mouse macrophages do not permit replication of the *C. burnetii* Nine Mile phase II (NMII) strain. However, eliminating TLR2 allows *C. burnetii* NMII to replicate within B6 macrophages, implying that innate immune pathways enable B6 macrophages to restrict *C. burnetii* replication. Whether other TLR signaling components or cytosolic immune pathways also sense and restrict *C. burnetii* NMII replication in mouse macrophages remains poorly understood. Here, we found that the TLR signaling adaptors MyD88 and Trif are required for cytokine production and act in concert to restrict *C. burnetii* NMII replication. Although we found that both TLR2 and TLR4 contribute to proinflammatory cytokine responses to *C. burnetii* NMII, TLR4 is dispensable for control of NMII replication. In addition, we did not observe a role for cytosolic sensing of *C. burnetii* T4SS activity in inflammasome activation or production of proinflammatory cytokines and type I interferons, nor did these pathways restrict *C. burnetii* NMII replication.

Introduction

To initiate innate immune defense and cell-intrinsic control of intracellular bacterial pathogens, host cells utilize pattern recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs) (Janeway, 1989; Janeway and Medzhitov, 2002; Medzhitov, 2007). Toll-like receptors (TLRs) located at the cell surface and within

endosomes detect extracellular PAMPs such as bacterial lipoproteins and lipopolysaccharide (LPS) (Akira, 2003). Downstream of TLRs, the adaptor molecules MyD88 and Trif activate several signaling pathways, including nuclear factor-kappa B (NF- κ B), mitogen-activated protein kinases (MAPKs), and interferon regulatory factor 3 (IRF3), which induce expression of proinflammatory cytokines and other anti-microbial effectors important for bacterial clearance (Akira, 2003). Cytosolic PRRs, such as those of the Nucleotide Binding Domain/Leucine Rich Repeat (NLR) and RIG-I-like receptor (RLR) families, respond to PAMPs introduced into the host cell cytosol by pore-forming toxins or specialized secretion systems of intracellular bacterial pathogens (Fritz et al., 2006; Inohara et al., 2005; Ting et al., 2006). Many cytosolic PRRs synergize with TLR signaling for increased proinflammatory cytokine production (Fritz et al., 2006; Oviedo-Boyso et al., 2014). In addition, a subset of cytosolic PRRs initiate assembly of a multiprotein complex termed the inflammasome, which activates the host proteases caspase-1 and caspase-11 (Martinon et al., 2002; Miao et al., 2010; Rathinam et al., 2012). Active caspase-1 initiates a form of cell death known as pyroptosis, a form of cell death that can restrict intracellular bacterial infection (Miao et al., 2010; Moltke et al., 2013; Rathinam et al., 2012). Pyroptosis is accompanied by release of IL-1 family cytokines (Miao et al., 2010; Moltke et al., 2013; Rathinam et al., 2012). Active caspase-11 also induces pyroptosis and IL-1 release (Broz et al., 2012; Gurung et al., 2012; Kayagaki et al., 2011; Rathinam et al., 2012). Together, these innate immune pathways collaborate to restrict many intracellular bacterial pathogens through cell-intrinsic and extrinsic mechanisms.

Coxiella burnetii is a facultative intracellular, gram-negative bacterium responsible for the zoonotic disease Q (query) fever, an acute flu-like illness that can progress to a

severe, chronic disease that often manifests as severe endocarditis (Maurin and Raoult, 1999). *C. burnetii* exists as two phase variants. Virulent phase I *C. burnetii* synthesizes LPS with a highly branched O-chain, which shields the bacteria from complement-mediated killing in serum and prevents binding of antibodies to cell surface (Hackstadt, 1988; Vishwanath and Hackstadt, 1988). In contrast, lab-adapted avirulent phase II *C. burnetii* produce a truncated O-antigen polysaccharide and are trafficked to fully mature lysosomes in B6 macrophages (Amano et al., 1987; Howe et al., 2010; Moos and Hackstadt, 1987). A cloned phase II variant of the *C. burnetii* Nine Mile reference strain (NMII; RSA493 clone 4) contains a ~26-kb chromosomal deletion that eliminates several genes involved in LPS biosynthesis and is avirulent in immunocompetent mice and guinea pigs (Hoover et al., 2002; Moos and Hackstadt, 1987). NMII is a biosafety level 2 organism, whereas all other *C. burnetii* strains require biosafety level 3. Although the NMII strain is unable to establish systemic infection in animals, the NMII strain and the isogenic phase I strain (NMI) are indistinguishable in their ability to replicate in human and mouse macrophage cell lines and primary human macrophages *in vitro* (Baca et al., 1981; Graham et al., 2013; Howe et al., 2010). There is no difference in the ability of NMI and NMII to elicit the proinflammatory cytokines TNF and IL-6 from human macrophages, but there are differences in the induction of other cytokines such as IL-1 β , suggesting that macrophages mount both overlapping and distinct immune responses to NMI and NMII during *in vitro* infection (Graham et al., 2013; Howe et al., 2010).

Once inside host cells, the *C. burnetii*-containing vacuole is directed down the canonical endosomal pathway, while also engaging the autophagy and early secretory pathways to form a lysosome-derived vacuole. (Heinzen et al., 1996; Romano et al., 2007) Within this compartment, *C. burnetii* is able to resist the bactericidal activities of

the lysosome and replicate for several days (Coleman et al., 2004; Heinzen et al., 1996). To establish this unique intracellular niche, *C. burnetii* utilizes a Dot/Icm type IVB secretion system to translocate over 100 effector proteins into the host cytosol (Beare et al., 2011; Carey et al., 2011; Chen et al., 2010; Graham et al., 2015; Lifshitz et al., 2013; Pan et al., 2008; Voth et al., 2011; 2009). These effector proteins are predicted to target and manipulate host cell factors, with several effectors established as having roles in intracellular membrane trafficking and inhibition of host cell apoptosis (Graham et al., 2015; Klingenberg et al., 2013; Lührmann and Roy, 2007; van Schaik et al., 2013).

The evolutionarily related pathogen *Legionella pneumophila*, which causes the severe pneumonia Legionnaires' disease, primarily associates with freshwater amoebae (Fields, 1996). Because *L. pneumophila* has evolved to infect protozoa, it is thought that *L. pneumophila* has not evolved evasion strategies to purposely subvert mammalian innate immune pathways and thus has been a useful model for uncovering innate immune pathways used by macrophages to restrict intracellular bacterial infection (Shin and Roy, 2008). In support of this, whereas A/J macrophages are permissive for *L. pneumophila* replication, C57BL/6 macrophages restrict *L. pneumophila* replication (Yoshida et al., 1991). Studying how C57BL/6 macrophages control *L. pneumophila* replication has allowed for the identification of TLR-dependent and cytosolic PRR-dependent immune pathways that collaborate to restrict *L. pneumophila* replication, such as the proinflammatory cytokines TNF and type I IFNs and inflammasome-dependent pyroptotic cell death. Importantly, activation of the inflammasome and other cytosolic immune pathways requires a functional *L. pneumophila* Dot/Icm T4SS that translocates bacterial products, such as flagellin, into the host cell cytosol.

Primary mouse macrophages of the C57BL/6 genetic background, in contrast to other inbred backgrounds, are not permissive for *C. burnetii* NMII infection (Zamboni, 2004; Zamboni et al., 2002). In contrast to *L. pneumophila*, how B6 mouse macrophages detect *C. burnetii* NMII and restrict intracellular replication of NMII remains poorly understood. TLR2 plays a major role in the production of proinflammatory cytokines and elimination of TLR2 signaling renders C57BL/6 macrophages more permissive for intracellular *C. burnetii* NMII replication (Zamboni et al., 2004). This indicates that innate immune sensing plays a critical role in restricting NMII replication in C57BL/6 macrophages. Furthermore, these findings suggest that C57BL/6 macrophages are a useful model for uncovering cell-intrinsic immune mechanisms that control intracellular *C. burnetii* NMII replication.

In contrast to TLR2, TLR4 is thought to be dispensable for immune responses against *C. burnetii*, as *C. burnetii* lipid A is a TLR4 antagonist (Shannon et al., 2005; Zamboni et al., 2004). Whether other TLRs might work in concert with TLR2 to detect and control *C. burnetii* NMII infection is unknown. Similarly, the roles of the TLR signaling adaptors MyD88 and Trif in mediating macrophage sensing and control of *C. burnetii* NMII is also unknown. Since *C. burnetii* encodes a Dot/Icm T4SS that translocates bacterial products into the host cell cytosol, it would be predicted that intracellular PRRs are activated that would restrict NMII replication (Beare et al., 2009; Newton et al., 2013; Segal et al., 2005; van Schaik et al., 2013). *C. burnetii* NMII is capable of translocating T4SS effectors into C57BL/6 macrophages, indicating that the block in bacterial replication may occur at a step following T4SS effector translocation (Newton et al., 2013). Indeed, the cytosolic sensor NOD2 was shown to contribute to cytokine production during infection with *C. burnetii* phase I organisms, demonstrating the potential for *C. burnetii* to

be detected by cytosolic surveillance pathways (Ammerdorffer et al., 2014; Benoit et al., 2009). Whether cytosolic immune pathways detect and restrict *C. burnetii* NMII infection in B6 macrophages is not known.

Here, we set out to identify whether additional TLR or cytosolic immune signaling pathways are used by C57BL/6 mouse macrophages to detect and restrict *C. burnetii* NMII during *in vitro* infection. In addition to the established role for TLR2 in responding to NMII and mediating proinflammatory cytokine production, we find that TLR2 and TLR4 work in concert to mediate cytokine responses to NMII, but we observe that TLR4 does not significantly contribute to the control of NMII replication. Furthermore, the TLR signaling adaptors MyD88 and Trif are both required for cytokine responses to NMII and restricting intracellular bacterial replication. Despite the ability of the *C. burnetii* NMII T4SS to translocate bacterial products into the cytosol of B6 macrophages, we did not observe inflammasome activation or an increase in pro-inflammatory cytokine production in response to *C. burnetii* NMII compared to isogenic mutants lacking a functional T4SS. In addition, the key inflammasome caspases, caspase-1 and caspase-11, were dispensable for control of intracellular NMII replication within B6 macrophages. We also did not detect robust induction of type I interferons by NMII, nor did we see a role for type I IFNs in restricting intracellular replication.

Materials and Methods

Ethics statement

All experiments performed in this study involving mice, solely used as a tissue source, were done so in accordance with the Animal Welfare Act (AWA) and the recommendations in the Guide for the Care and Use of Laboratory Animals of the

National Institutes of Health. The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all procedures (protocols #803465, #803459, #804714, and #804928).

Bacteria Strains

Coxiella burnetii Nine Mile Phase II (clone 4; RSA 439) and *Legionella pneumophila* serogroup 1 background strains were used in all experiments (Berger and Isberg, 1993). For *C. burnetii* infections, acidified citrate cysteine medium (ACCM-2) was inoculated with WT *C. burnetii* (Omsland et al., 2013), WT *C. burnetii* expressing mCherry (Beare et al., 2009), *icmL*::Tn strain (Carey et al., 2011), which contains a transposon insertion in the *icmL* gene rendering the T4SS non-functional, and WT and *icmL*::Tn strains harboring a plasmids encoding BlaM alone and BlaM fused to known T4SS substrate CBU_0077 under control of the CBU_1169 promoter (Carey et al., 2011; Newton et al., 2013). *C. burnetii* was grown at 37°C in 5% CO₂ and 2.5% O₂ for 6 days to late log phase (~1.0x10⁹ bacteria/mL). One day after inoculation, kanamycin (275 µg/mL) was added to *icmL*::Tn cultures and chloramphenicol (3 µg/mL) was added to strains harboring plasmids encoding BlaM and BlaM-0077 fusion proteins (Carey et al., 2011). To quantify *C. burnetii* genome equivalents for infection of macrophages, *C. burnetii* genomic DNA was isolated with the illustra™ bacteria genomicPrep Mini Spin Kit (GE Healthcare), and genomic equivalents were measured by quantitative PCR of the *C. burnetii dotA* gene using SYBR Green, the CFX96 qPCR machine (Bio-Rad Laboratories), and the following primers: *dotA* 5' (GCGCAATACGCTCAATCACA), *dotA* 3' (CCATGGCCCCAATTCTCT). *L. pneumophila* $\Delta dotA$ (Berger et al., 1994), $\Delta flaA$ (Ren et al., 2006), and $\Delta dotA$ and $\Delta flaA$ harboring the pSS128 encoding a *blam*-RalF

fusion plasmid on the Lp02 (*thyA*) background, a thymidine auxotroph derived from strain Lp01, were cultured as a heavy patch on charcoal yeast extract agar containing thymidine for 48 hours at 37°C (Copenhaver et al., 2014). *L. pneumophila* and *C. burnetii* strains harboring plasmids encoding the BlaM reporter proteins were grown in the presence of chloramphenicol at 6.75 µg/mL and 3 µg/mL, respectively.

Mammalian Cell Culture

Bone marrow was isolated from the femurs, tibia, and humeri of C57BL/6 (Jackson), *Tlr2*^{-/-} (Takeuchi et al., 1999), *Tlr4*^{-/-} (Hoshino et al., 1999), *Tlr2*^{-/-}*Tlr4*^{-/-}, *Myd88*^{-/-} (Adachi et al., 1998), *Trif*^{-/-} (Yamamoto et al., 2003), *Myd88*^{-/-}*Trif*^{-/-}, and *Ifnar*^{-/-} (Müller et al., 1994) mice. Bone marrow cells were differentiated into macrophages for 7-8 days in RPMI containing 30% L929 cell supernatant and 20% FBS at 37°C in a humidified incubator (Casson et al., 2013). Macrophages were lifted with ice-cold PBS + 2 mM EDTA and replated one day prior to infection in RPMI containing 15% L929 cell supernatant and 10% FBS. Macrophages were plated into 48 or 24 well plates at 1.5x10⁵ or 2.0x10⁵ cells per well, respectively. Macrophages were infected with various MOIs of *C. burnetii* and *L. pneumophila* as indicated. *C. burnetii* and *L. pneumophila* bacteria were washed once with PBS prior to infection of macrophages. After infection, cells were spun at 1200 RPM for 5 minutes prior to incubation at 37°C. At designated timepoints, macrophage supernatants, whole cell lysates, or mRNA were collected.

Immunoblot Analysis

Cell lysates were collected with 1X SDS-PAGE sample buffer or supernatants were mixed 1:1 with 2X SDS-PAGE buffer containing protease and phosphatase inhibitors.

Samples were boiled for 5 minutes, separated by SDS-PAGE and transferred to Immobilon P membranes (Millipore). Primary antibodies specific for phospho-p38 and p38 MAPK (Cell Signaling Technology), caspase-1 p10 (Santa Cruz Biotechnology), IL-1 β (R&D Systems), and β -actin (Sigma) were used. Detection was performed with HRP-conjugated anti-rabbit antibodies (Cell Signaling Technology), anti-mouse antibodies (Cell Signaling Technology), or anti-rat antibodies (Jackson ImmunoResearch).

ELISA

Harvested supernatants from infected macrophages were assayed for cytokines using capture and detection antibodies specific for TNF, IL-6, IL-1 α , and IL-1 β (BD Biosciences).

Cytotoxicity Assay

Cells were infected with WT *C. burnetii* for 24 hours or treated with 0.5 μ g/mL LPS for 4 hours and 2.5 mM ATP for 1 hour. Supernatants were harvested and used to measure % cytotoxicity via lactate dehydrogenase (LDH) release using the LDH Cytotoxicity Assay Kit (Clontech).

Measuring Intracellular *Coxiella burnetii* Replication

Measuring intracellular replication of *C. burnetii* in C57BL/6, *Tlr2*^{-/-}, *Tlr4*^{-/-}, *Tlr2*^{-/-}*Tlr4*^{-/-}, *Myd88*^{-/-}, *Trif*^{-/-}, *Myd88*^{-/-}*Trif*^{-/-}, or *Ifnar*^{-/-} BMDMs was performed as follows. Macrophages were infected with *C. burnetii* Nine Mile Phase II expressing mCherry at MOI=100 in 24 well plates (Beare et al., 2011). 24 hours post-infection, cells were washed 3 times with PBS to remove extracellular bacteria and fresh media was added to the cells every 2

days. At designated timepoints post-infection, the extracellular media was collected and the adherent macrophages were lysed with 1mL sterile dH₂O. The lysed samples were then combined with the extracellular media from the same well. Bacterial genomic DNA was purified and *C. burnetii* genomic equivalents (GE) were measured via qPCR using primers specific for the *C. burnetii dotA* gene. The fold change in bacterial GEs was calculated as a ratio of the number of GEs on a given day to the number of GEs on day 1.

Enumeration of *Coxiella burnetii*-containing vacuoles by microscopy

2.0×10^5 cells were plated onto a glass coverslip per well in triplicate in 24 well plates for each condition. Seven days post-infection, cells were washed with PBS, stained with DAPI, and fixed with 4% paraformaldehyde. Images were taken with a Nikon Eclipse 2000E-U epifluorescence microscope and images were acquired with NIS Elements B4 4.10.01 software. Large vacuoles ($> 5 \mu\text{M}$) containing mCherry-expressing *C. burnetii* were enumerated as a percentage of total DAPI-positive cells. For each coverslip, greater than 300 cells were counted.

Quantitative RT-PCR

RNA was isolated from infected macrophages using the RNeasy Mini kit and DNase-treated using the RNase-free Dnase set (Qiagen) to remove contaminating genomic DNA. The isolated RNA was then reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen). Relative mRNA abundance was measured by qPCR using SYBR Green and the CFX96 qPCR machine (Bio-Rad Laboratories). The following primer pairs were used: *Ifnb* 5' (GCACTGGGTGGAATGAGACTATTG), *Ifnb* 3'

(TTCTGAGGCATCAACTGACAGGTC), *Ifna4* 5' (CCCACAGCCCAGAGAGTGACC), *Ifna4* 3' (GGCCCTCTTGTTCCCGAGGT), *Hprt* 5' (GTTGGATACAGGCCAGACT), *Hprt* 3' (GAGGGTAGGCTGGCCTAT). To calculate relative fold induction using the $\Delta\Delta\text{CT}$ method, the cycle threshold (Ct) of a given gene was normalized to HPRT Ct and compared to the normalized Ct in uninfected cells.

Viral Proliferation Bioassay

The viral proliferation bioassay was performed as previously described, with some modifications as follows (Park et al., 2003). Briefly, C57BL/6 macrophages were mock infected, treated with Poly (I:C) (Invivogen), or infected with *L. pneumophila* ΔflaA at MOI=5 or WT *C. burnetii* NMII at MOI=50 for 24 hours. Supernatants were collected and UV-inactivated by exposure to 600 mJoules·cm⁻² UVA light in a Stratalinker 1800 (Stratagene). L2 mouse fibroblasts were treated with UV-inactivated supernatants for 24 hours and then infected with Newcastle disease virus expressing green fluorescent protein (NDV-GFP) at an MOI of 1 plaque-forming unit (PFU)/cell. At 24 hours post-infection, cells were fixed, stained with DAPI, and examined under an Eclipse TE2000-U epifluorescence microscope (Nikon Instruments, Inc.). Images were acquired using NIS Elements B4 4.10.01 software (Nikon Instruments, Inc.). Mean GFP fluorescence intensity was determined using ImageJ, with background fluorescence determined from mock viral-infected samples. For each sample, the mean of three random fields of equal area was quantified. Images and quantification are representative of two independent experiments.

Effector Translocation Assay

Translocation of BlaM-effector fusion proteins was performed as previously described (Copenhaver et al., 2014; Newton et al., 2013). 5.0×10^5 BMDMs were seeded over night in 12 well non-TC coated dishes. Cells were infected at indicated MOIs. 16 hours post-infection, cells were loaded with the fluorescent substrate CCF4-AM, using the Live BLAzer-RET B/G Loading Kit with 15 mM probenecid, in the dark for 90 min at room temperature (Copenhaver et al., 2014; Newton et al., 2013). Cells were gated on live singlets that had retained the CCF4-AM dye.

Treatment of B6 Macrophages with Purified *Coxiella burnetii* Nine Mile phase II LPS

LPS from Nine Mile Phase II *C. burnetii* was purified as previously described (Zamboni et al., 2004) and resuspended in deionized water. C57BL/6 bone marrow derived macrophages were plated in 96 wells at 1.0×10^5 cells per well for a total volume of 100 μ L. Cells were treated with 0, 100, and 1000 ng/mL of *C. burnetii* for 24 hours. To ensure the purified LPS was present, we tested its previously described ability to antagonize *E. coli* LPS (Zamboni et al., 2004). 30 minutes post addition of *C. burnetii* LPS, we added 0, 0.1, 1, and 10 ng/mL *E. coli* LPS. Cells were incubated for 24 hours and TNF levels were measured via ELISA.

Statistical Analysis

The plotting of data and statistical analysis were performed using GraphPad Prism software. Statistical significance was determined using unpaired, two-tailed Student's *t* test or one-way analysis of variance (ANOVA) with Tukey's posttest. Differences were considered significant if the *P* value was <0.05 .

Results

Both TLR2 and TLR4 mediate cytokine responses to *Coxiella burnetii* Nine Mile phase II in mouse macrophages.

Production of pro-inflammatory cytokines in response to *C. burnetii* infection has previously been reported to depend largely on TLR2 signaling (Ammerdorffer et al., 2014; Zamboni et al., 2004). However, the roles of other TLRs or the signaling adaptors MyD88 or Trif in macrophage responses to *C. burnetii* are largely unknown. In particular, it is unclear if TLR4 contributes to the immune response to *C. burnetii*. TLR4-deficient mice have a defect in cytokine production during *C. burnetii* infection *in vivo* (Honstetter et al., 2004), but the lipid A of *C. burnetii* is thought to be an antagonist of TLR4 and macrophages lacking a functional TLR4 do not exhibit a defect in cytokine production (Zamboni et al., 2004). Given that TLR2 responds so strongly to *C. burnetii*, we wondered whether a role for TLR4 could be revealed in the absence of TLR2 signaling. Thus, we infected B6, *Tlr2*^{-/-}, *Tlr4*^{-/-}, and *Tlr2*^{-/-}*Tlr4*^{-/-} bone marrow-derived macrophages (BMDMs) with *C. burnetii* NMII and measured secreted levels of TNF and IL-6 at 24 hours post infection. We observed a significant reduction in TNF and IL-6 production in infected *Tlr2*^{-/-} BMDMs and no difference in cytokine production in infected *Tlr4*^{-/-} BMDMs, consistent with previous published studies (Figures 2.1A and 2.1B). However, BMDMs lacking TLR2 still produced substantial levels of TNF and IL-6 following *C. burnetii* infection at an MOI of 50, indicating that an additional TLR also contributes to cytokine production. In contrast, BMDMs lacking both TLR2 and TLR4 did not produce detectable levels of TNF and IL-6 in response to *C. burnetii* infection at an MOI of 50, indicating a role for TLR4 in sensing *C. burnetii* that is unmasked in the absence of TLR2.

B6 BMDMs do not permit robust intracellular *C. burnetii* replication (Zamboni, 2004). Restriction of *C. burnetii* replication is in part mediated through TLR2 signaling, as TLR2-deficient BMDMs are significantly more permissive for *C. burnetii* replication (Zamboni et al., 2004). To test if restriction of *C. burnetii* replication also involves TLR4, we infected B6, *Tlr2*^{-/-}, *Tlr4*^{-/-}, and *Tlr2*^{-/-}*Tlr4*^{-/-} BMDMs with WT *C. burnetii* expressing mCherry and measured bacterial uptake (Figure 2.8A) on day 1 post-infection, and intracellular replication (Figure 2.1C) and vacuole formation (Figures 2.1D and 2.1E) at day 7 post-infection. Similar levels of bacterial uptake were observed for the different genotypes of mouse macrophages. In agreement with previous findings, B6 and *Tlr4*^{-/-} BMDMs limited bacterial replication, whereas TLR2-deficient BMDMs were more permissive for intracellular *C. burnetii* replication. *Tlr2*^{-/-}*Tlr4*^{-/-} BMDMs were not more permissive for *C. burnetii* replication when compared to *Tlr2*^{-/-} BMDMs, suggesting that concomitant deletion of both TLR2 and TLR4 does not increase macrophage susceptibility to *C. burnetii* replication, despite decreased cytokine production.

The TLR signaling adaptors MyD88 and Trif are critical for cytokine production and cell-intrinsic control of intracellular *Coxiella burnetii* replication in mouse macrophages.

Because TLR2 primarily signals through the adaptor protein MyD88, whereas TLR4 signals through both MyD88 and Trif (Akira, 2003), we next examined the roles of MyD88 and Trif in mediating cytokine responses to *C. burnetii*. *Myd88*^{-/-} BMDMs produced significantly lower, but still substantial, levels of TNF and IL-6 compared to WT BMDMs following *C. burnetii* infection at an MOI of 50 (Figures 2.2A and 2.2B). *Trif*^{-/-} BMDMs similarly produced significantly lower, but substantial, levels of TNF and IL-6

compared to WT BMDMs. In contrast, *Myd88*^{-/-}*Trif*^{-/-} BMDMs did not produce detectable levels of TNF or IL-6, suggesting that both MyD88 and Trif signaling contribute to cytokine production during *C. burnetii* infection. Thus, in addition to confirming that TLR2 is a major sensor of *C. burnetii*, these data implicate a role for TLR4 and/or another Trif-dependent TLR in responding to *C. burnetii* NMII and mediating cytokine production.

To test if MyD88 and Trif also contribute to the restriction of *C. burnetii* replication, we infected B6, *Myd88*^{-/-}, *Trif*^{-/-}, and *Myd88*^{-/-}*Trif*^{-/-} BMDMs with WT *C. burnetii* expressing mCherry and measured bacterial uptake (Figure 2.8B) on day 1 post-infection as well as intracellular replication (Figure 2.2C) and vacuole formation (Figures 2.2D and 2.2E) at day 7 post-infection. Similar levels of bacterial uptake were observed for the different genotypes of BMDMs. When we examined the levels of intracellular *C. burnetii* replication at day 7 post-infection, we found that *Myd88*^{-/-} BMDMs were significantly more permissive than B6 BMDMs, whereas *Trif*^{-/-} BMDMs did not exhibit increased bacterial growth. BMDMs deficient in both Myd88 and Trif were more permissive than MyD88-deficient BMDMs for intracellular *C. burnetii* growth, as measured by both increased GEs and vacuole number. These data indicate that both MyD88 and Trif mediate cytokine production and restriction of intracellular *C. burnetii* replication in B6 BMDMs.

Purified *Coxiella burnetii* LPS does not induce TNF production.

Since we observed a role for TLR4 in the production of TNF, we wanted to test if purified *C. burnetii* Nine Mile Phase II LPS was capable of stimulating TNF production in our system. C57BL/6 failed to produce TNF in response to two concentrations of *C. burnetii* LPS, 100 ng/mL or 1000 ng/mL (Figure 2.3). To confirm our sample contained functional *C. burnetii* LPS, we performed an LPS antagonism assay, as *C. burnetii* LPS

has been reported to be antagonistic (Zamboni et al., 2004). Cells were pretreated with *C. burnetii* LPS and then stimulated with different doses of *E. coli* LPS ranging from 0.1 to 10 ng/mL (Zamboni et al., 2004). We saw that *C. burnetii* LPS antagonized the stimulatory activity of *E. coli* LPS similarly to the previous study (Figure 2.3). These data suggest that *C. burnetii* LPS alone fails to stimulate TLR4. TLR4-induced TNF production could require a second signal from TLR2 or may be stimulated by another yet-to-be-identified bacterial or host ligand.

***Coxiella burnetii* translocates effector proteins into B6 macrophages.**

To confirm that *C. burnetii* in our system was capable of translocating bacterial products into the host cell cytosol, we used a β -lactamase reporter system to measure T4SS effector translocation. As positive and negative controls, we used the *L. pneumophila* $\Delta flaA$ strain and $\Delta dotA$ strain, which lack a functional T4SS, both harboring a plasmid encoding a translational fusion of the *E. coli* TEM-1 β -lactamase (BlaM) and the T4SS effector RalF. RalF is a *L. pneumophila* effector that is robustly translocated into host cells (Copenhaver et al., 2014). For *C. burnetii*, we used WT and *icmL::Tn* Nine Mile Phase II carrying a plasmid encoding BlaM translationally fused to CBU_0077, a *C. burnetii* effector protein previously shown to be translocated into macrophages at relatively early time points, by 4 to 8 hours post-infection (Newton et al., 2013). Translocation of the fusion proteins was detected in host cells by means of the membrane-permeable BlaM substrate CCF4-AM (Zlokarnik et al., 1998). CCF4-AM consists of coumarin joined to fluorescein by a β -lactam ring. When excited at 409nm, fluorescence resonance energy transfer (FRET) between coumarin and fluorescein results in green fluorescence emission at 518nm. T4SS-injected BlaM will cleave the

CCF4-AM substrate in the host cytosol and eliminate FRET, thus resulting in blue fluorescence emission at 447nm. As expected, the *L. pneumophila* $\Delta flaA$ strain robustly translocated RalF into macrophages, whereas the $\Delta dotA$ strain was unable to translocate (Figure 2.4). At 16 hours post-infection, with the Nine Mile Phase II strains expressing BlaM-0077, we were able to see dose-dependent injection into C57BL/6 macrophages infected with WT, but not *icmL::Tn* (Figure 2.4). This confirms that in our system, *C. burnetii* is capable of introducing bacterial products into the cytosol of replication-restrictive C57BL/6 macrophages.

***Coxiella burnetii* T4SS activity does not enhance cytokine production.**

Cytosolic sensing of bacterial products translocated into the host cell cytosol by specialized secretion systems can contribute to increased cytokine production and restriction of bacterial replication. In the case of *L. pneumophila*, cytosolic sensing of T4SS-translocated products leads to enhanced cytokine production (Shin et al., 2008). *C. burnetii* NMII is capable of translocating T4SS effectors into C57BL/6 macrophages (Newton et al., 2013), but whether cytosolic sensing of *C. burnetii* T4SS activity might similarly lead to increased pro-inflammatory cytokine production is unknown. To test this possibility, we infected WT macrophages with WT *C. burnetii* or T4SS-deficient *C. burnetii* (*icmL::Tn*) containing an inactivating transposon insertion in the *icmL* gene (Carey et al., 2011). We then measured production of pro-inflammatory cytokines TNF and IL-6. As a positive control for T4SS-dependent cytokine production, we used *L. pneumophila* $\Delta flaA$, which express a functional T4SS but lack flagellin to bypass NAIP5 inflammasome activation and host cell death in B6 BMDMs (Ren et al., 2003), or *L. pneumophila* $\Delta dotA$, which lack a functional T4SS (Berger et al., 1994). As previously

demonstrated, *L. pneumophila* $\Delta flaA$ infection induced increased production of TNF and IL-6 compared to infection with *L. pneumophila* $\Delta dotA$. In contrast, we did not observe enhanced TNF or IL-6 production in BMDMs infected with WT *C. burnetii* compared to infection with *C. burnetii* *icmL::Tn* in restrictive C57BL/6 or permissive *Tlr2^{-/-}* (Figures 2.5A-D). Moreover, in contrast to increased T4SS-dependent expression of pro-IL-1 β in response to *L. pneumophila* $\Delta flaA$ compared to $\Delta dotA$ infection, we did not observe differential expression of pro-IL-1 β between WT and *C. burnetii* *icmL::Tn* (Figure 2.6H). These findings suggest cytosolic sensing of *C. burnetii*'s T4SS does not lead to increased production of the pro-inflammatory cytokines TNF, IL-6, or pro-IL-1 β . We did, however, consistently observe a slight decrease in cytokine production in cells infected with WT *C. burnetii* compared to cells infected with *icmL*-deficient bacteria, suggesting that *C. burnetii* T4SS activity may somehow diminish production of pro-inflammatory cytokines.

Early p38 MAPK activation in response to *Coxiella burnetii* infection is mediated by TLR signaling.

Upon detection of PAMPs by surface and cytosolic PRRs, signaling events that involve phosphorylation and activation of host MAPKs contribute to the generation of a pro-inflammatory transcriptional response (Janeway and Medzhitov, 2002). For example, *L. pneumophila* induces rapid p38 MAPK activation within 20 minutes post-infection that involves both TLR signaling as well as cytosolic sensing of T4SS-translocated bacterial effectors (Fontana et al., 2012; Shin et al., 2008). p38 MAPKs are activated and contribute to cytokine production during *C. burnetii* infection, but the mechanistic basis for MAPK activation in response to *C. burnetii* is unknown (Ammerdorffer et al., 2014;

Barry et al., 2012; Voth and Heinzen, 2009). To test if p38 MAPK phosphorylation shortly after *C. burnetii* infection requires TLR signaling, we infected B6 and *Myd88*^{-/-} BMDMs with WT *C. burnetii* and examined p38 MAPK phosphorylation up to two hours post-infection (Figure 2.5E). We observed robust phosphorylation of p38 MAPK in B6 macrophages, but p38 MAPK phosphorylation was attenuated in MyD88-deficient BMDMs, suggesting that early p38 MAPK activation in response to *C. burnetii* is largely TLR-dependent and is not robustly triggered by cytosolic immunosurveillance pathways.

Inflammasomes are not activated by *Coxiella burnetii* in mouse macrophages and do not restrict intracellular replication.

Bacterial ligands introduced into the host cell cytoplasm by virulence-associated specialized secretion systems can be sensed by cytosolic PRRs (Fritz et al., 2006; Inohara et al., 2005; Ting et al., 2006). A subset of cytosolic PRRs trigger the assembly of a multi-protein cytosolic complex termed the inflammasome. Inflammasomes recruit and activate the host proteases caspase-1 and caspase-11, which regulate an inflammatory cell death termed pyroptosis and promote the release of IL-1 family cytokines, including IL-1 α , IL-1 β , and IL-18 (Martinon et al., 2002; Miao et al., 2010; Rathinam et al., 2012). This inflammatory cell death can restrict intracellular bacterial replication and contributes to a systemic *in vivo* immune response involving neutrophil recruitment and IFN γ production and subsequent bacterial clearance. Infection with T4SS-expressing *L. pneumophila* leads to robust activation of multiple inflammasomes, including a flagellin-dependent NAIP5 inflammasome, an NLRP3 inflammasome that responds to an unidentified T4SS-dependent signal, and a caspase-11 inflammasome that detects hexa-acylated or penta-acylated lipid A (Case and Roy, 2011; Case et al.,

2013; 2009; Casson et al., 2013; Hagar et al., 2013; Kayagaki et al., 2011; Molofsky, 2006; Ren et al., 2006; Shi et al., 2014; Zamboni et al., 2006). *C. burnetii* does not contain genes encoding flagellin and thus would not be expected to activate the NAIP5 inflammasome (Seshadri et al., 2003). Furthermore, *C. burnetii* expresses tetra-acylated lipid A, which is thought to be non-stimulatory for caspase-11, and thus would not be expected to activate the caspase-11 inflammasome (Hagar et al., 2013; Kayagaki et al., 2011; Zamboni et al., 2004). However, whether *C. burnetii* infection induces activation of the NLRP3 inflammasome or other inflammasomes is unknown. To test if *C. burnetii* infection of mouse macrophages leads to inflammasome activation, we first infected B6 BMDMs with *C. burnetii* and measured secretion of the inflammasome-dependent cytokines IL-1 α and IL-1 β , as well as cell death. As a positive control for inflammasome activation, we infected BMDMs with *L. pneumophila* Δ *flaA*, which lack flagellin and are unable to activate the NAIP5 inflammasome but still activate the NLRP3 and caspase-11 inflammasomes. As expected, infection with *L. pneumophila* Δ *flaA* at an MOI of 5 induced robust release of both IL-1 α and IL-1 β (Figures 2.6A and B). In contrast, we were unable to detect released IL-1 α and IL-1 β in the supernatant of *C. burnetii*-infected cells even at a high MOI of 100 at 24 hours post-infection, suggesting that *C. burnetii* infection fails to induce inflammasome-dependent cytokine secretion (Figures 2.6A and 2.6B). Furthermore, we did not detect cell death 24 hours post-infection in *C. burnetii*-infected cells, whereas treatment with the NLRP3 inflammasome stimulus LPS + ATP induced robust cell death (Figure 2.6C).

Inflammasome activation is a two step process that requires an initial ‘priming’ signal, often TLR signaling, to upregulate the expression of inflammasome components, including pro-IL-1 β , followed by a second ‘activation’ signal involving cytosolic detection

of bacterial PAMPs (Fontana and Vance, 2011). WT *C. burnetii* infection is capable of priming macrophages, as measured by detection of pro-IL-1 β in the lysates of B6 BMDMs infected with WT *C. burnetii* at MOIs ranging from 20 to 100 (Figure 2.6G); however, the levels of pro-IL-1 β induced in *C. burnetii*-infected B6 BMDMs even at an MOI of 100 were substantially lower than in BMDMs infected with *L. pneumophila* at an MOI of 5, indicating that *C. burnetii* does not prime BMDMs as robustly as *L. pneumophila*. Thus, it is possible that the level of priming induced by *C. burnetii* infection is insufficient for inflammasome activation and subsequent IL-1 α and IL-1 β release. To test this possibility, we next pre-primed B6 BMDMs with *E. coli* LPS for 4 hours, an established method of inflammasome priming that induces robust expression of pro-IL-1 β and other inflammasome components (Casson et al., 2013). We then infected LPS-primed cells with *L. pneumophila* Δ *flaA* or WT *C. burnetii* NMII and measured secreted IL-1 α and IL-1 β (Figures 2.6D and 2.6E). Mock-infected, LPS-primed BMDMs did not release IL-1 α or IL-1 β into the supernatant, as LPS-priming alone is insufficient for inflammasome activation, whereas *L. pneumophila* Δ *flaA*-infected primed BMDMs released substantial levels of IL-1 α and IL-1 β by 24 hours post-infection. In contrast, we were unable to detect secreted IL-1 α or IL-1 β in the supernatants of LPS-primed cells infected with WT *C. burnetii* at MOIs ranging from 20 to 100 for 24 hours, suggesting that even following LPS-priming, *C. burnetii* NMII infection fails to induce inflammasome-dependent cytokine secretion. We next examined whether the failure to detect inflammasome-dependent cytokine secretion was due to an upstream defect in caspase-1 activation by assaying for caspase-1 processing into a mature 10kD-sized fragment (p10) and its release into the supernatant of infected cells (Figure 2.6H) Mock-infection of LPS-primed BMDMs did not result in caspase-1 processing and release, whereas *L.*

pneumophila $\Delta flaA$ infection led to caspase-1 processing into a detectable p10 form. In contrast, caspase-1 processing was undetectable following WT *C. burnetii* infection at all MOIs tested. These data indicate that *C. burnetii* NMII infection can lead to the upregulation of inflammasome-related genes such as pro-IL-1 β , but *C. burnetii* T4SS activity does not trigger inflammasome activation following infection of mouse macrophages. Moreover, despite similar levels of internalized bacteria at 24 hours post-infection (Figure 2.8C), we did not detect increased *C. burnetii* replication in *Casp1*^{-/-} *Casp11*^{-/-} BMDMs, in contrast to *Tlr2*^{-/-} BMDMs (Figure 2.6F), further demonstrating that inflammasomes do not play a critical role in controlling intracellular *C. burnetii* replication in B6 BMDMs.

Type I interferons are not robustly induced by *Coxiella burnetii* and do not restrict intracellular replication in mouse macrophages.

Many intracellular bacterial pathogens stimulate the production of type I interferon (IFN) in infected host cells (Monroe et al., 2010). Induction of type I IFNs is regulated in part by extracellular sensors, such as TLR4, as well as cytosolic sensors, such as the RNA sensors RIG-I and MDA-5, the DNA sensor cGAS, or the cyclic dinucleotide sensor STING (Burdette and Vance, 2013; Dempsey and Bowie, 2015; Monroe et al., 2010; Paludan, 2015). In some bacterial infections, such as *L. pneumophila*, type I IFNs promote bacterial clearance (Coers et al., 2007; Lippmann et al., 2011; Schiavoni et al., 2004), while in other infections, such as *M. tuberculosis*, activation of type I IFNs is detrimental to the host (Manzanillo et al., 2012). Whether *C. burnetii* induces type I IFNs upon infection of macrophages is unknown. To test if *C. burnetii* activates a type I IFN response, we infected B6 BMDMs with WT *C. burnetii* and measured levels of *Ifna4* and

Ifnb mRNAs by quantitative RT-PCR (Figures 2.7A and 2.7B). We were unable to detect transcriptional induction of IFN α 4 or IFN β 16 hours post infection, suggesting that *C. burnetii* does not robustly induce expression of type I IFNs. In contrast, we observed robust upregulation of *Ifna4* and *Ifnb* mRNAs in response to *L. pneumophila* Δ *flaA* infection (Figures 2.7A and 2.7B). However, it is possible that undetectable but biologically active amounts of type I IFNs are still produced during infection. Alternatively, it is possible that *C. burnetii* may induce expression of one of the other nine known type I IFNs. We therefore used a bioassay that detects type I IFN antiviral activity by measuring the ability of supernatants from *C. burnetii*-infected BMDMs to inhibit replication of GFP-expressing Newcastle disease virus (NDV) in mouse fibroblasts (Park et al., 2003). As expected, supernatants from BMDMs treated with the TLR3 ligand poly(I:C) or *L. pneumophila* Δ *flaA*-infected macrophages, which both induce robust type I IFN production, markedly decreased viral replication as measured by decreased GFP intensity compared to mock-treated cells, confirming that these two stimuli induce IFN production (Figures 2.7C and 2.7D). Supernatants from *C. burnetii* NMII-infected BMDMs also led to decreased viral replication, although to a lesser degree, suggesting that low, biologically active levels of type I IFN are produced during *C. burnetii* infection. Since type I IFN signaling restricts the intracellular replication of *L. pneumophila* in BMDMs (Coers et al., 2007), we next tested if type I IFN signaling played a similar role in the restriction of *C. burnetii* by measuring replication of WT *C. burnetii* in B6 or *Ifnar*^{-/-} BMDMs, as all type I IFNs signal through the type I IFN receptor (IFNAR) (Müller et al., 1994). IFNAR-deficient BMDMs did not exhibit increased *C. burnetii* replication by day 7 post-infection (Figure 2.7E). These data demonstrate that *C. burnetii* does not robustly

induce production of type I IFNs, nor are type I IFNs required to restrict intracellular *C. burnetii* replication in B6 BMDMs.

Discussion

Coxiella burnetii can infect and replicate within macrophages, yet little is known about the immune mechanisms utilized by macrophages to detect and restrict intracellular *C. burnetii* replication. In our study, we aimed to identify innate immune pathways used by B6 macrophages to detect and restrict *C. burnetii* NMII. B6 macrophages are not permissive for intracellular *C. burnetii* growth (Zamboni et al., 2004), despite the ability of *C. burnetii* to translocate T4SS effectors into these cells (Newton et al., 2013). Past studies identified a critical role for TLR2 in enabling B6 macrophages to detect and limit *C. burnetii* replication (Zamboni et al., 2004), suggesting that innate immunity plays a key role in controlling *C. burnetii* infection in this cell type. However, the roles of other TLRs and the signaling adaptors MyD88 and Trif in the innate immune response to *C. burnetii* remain poorly understood. Here, we find that TLR2 and TLR4 signal in concert to mediate proinflammatory cytokine production in response to *C. burnetii* NMII. The TLR signaling adaptors MyD88 and Trif were required for this cytokine response and also contributed to the restriction of *C. burnetii* replication. Furthermore, to date, whether cytosolic immunosurveillance pathways detect bacterial products translocated by the *C. burnetii* T4SS and limit *C. burnetii* growth in B6 macrophages is unclear. In this study, we find that *C. burnetii* does not induce inflammasome activation or robust production of proinflammatory cytokines or type I IFNs in a T4SS-dependent manner, suggesting that *C. burnetii* does not robustly activate cytosolic immune sensing pathways in B6 macrophages.

We first set out to examine the role of TLR signaling in pro-inflammatory cytokine production and bacterial restriction in B6 BMDMs infected with *C. burnetii* NMII. In agreement with previous findings (Zamboni et al., 2004), we found production of TNF and IL-6 to be largely mediated by TLR2. Interestingly, we also uncovered a role for TLR4 in the production of these cytokines. Deletion of TLR4 alone did not impact cytokine production, consistent with previous findings, but concomitant ablation of TLR2 and TLR4 yielded a significant decrease in cytokine production greater than that observed with TLR2 deficiency alone. Consistent with a role for both TLR2 and TLR4 in immune sensing of *C. burnetii* NMII, we found that the adaptor proteins MyD88, which signals downstream of both TLR2 and TLR4, and Trif, which signals downstream of TLR4, are both required for cytokine production and restriction of bacterial replication in infected macrophages. As we were able to uncover a role for TLR4 alone in the absence of TLR2, this suggests that TLR4 is not essential for responding to *C. burnetii*, consistent with previous findings showing that *C. burnetii* LPS is not stimulatory for TLR4 (Zamboni et al., 2004). However, it is possible in the context of the whole bacterium, TLR4 somehow responds to *C. burnetii*, either by sensing lipid A or another PAMP, but that TLR2 plays a more major role in sensing *C. burnetii*. As virulent phase I *C. burnetii*, in contrast to avirulent phase II *C. burnetii*, inhibits p38 MAPK signaling in a TLR4-dependent manner (Barry et al., 2012), further studies are necessary to test if TLR2 and TLR4 act in concert to respond to phase I *C. burnetii* as well. These studies could provide important insight into whether full-length LPS is an evasion strategy used by phase I *C. burnetii* to limit TLR detection. Furthermore, we found a dominant role for TLR signaling in mediating p38 MAPK activation in response to *C. burnetii* NMII infection.

These results show that production of cytokines in response to *C. burnetii* infection is highly dependent on TLR sensing.

TLRs detect extracellular PAMPs at the cell surface and within endosomes (Akira, 2003; Janeway, 1989; Janeway and Medzhitov, 2002; Medzhitov, 2007). Therefore, to mount an immune response tailored against intracellular bacterial pathogens (Fritz et al., 2006; Inohara et al., 2005; Ting et al., 2006), the immune system often relies on cytosolic PRRs that respond to bacterial products introduced into the host cytosol by many intracellular bacterial pathogens. Studies on the evolutionarily related pathogen *L. pneumophila* have shown that its Dot/Icm T4SS is required for translocation of bacterial effectors into the host cell cytosol and bacterial survival within macrophages, but also inadvertently exposes *L. pneumophila* to an array of cytosolic immunosurveillance pathways. Infection with WT *L. pneumophila* expressing a functional T4SS significantly increases the production of TNF, IL-6, and IL-12 and robustly activates host MAPK signaling, type I IFN production, and several inflammasome pathways compared to avirulent *L. pneumophila* mutants lacking a functional T4SS (Casson et al., 2013; Shin et al., 2008). Several of these pathways restrict intracellular *L. pneumophila* replication. *Coxiella burnetii* uses a closely related Dot/Icm T4SS to translocate effector proteins into the host cell cytosol, and *C. burnetii* NMII can translocate T4SS effectors into B6 BMDMs. Thus, we next asked whether cytosolic immunosurveillance pathways respond to *C. burnetii* T4SS activity and restrict bacterial replication in B6 BMDMs (Newton et al., 2013). In contrast to what we observed for *L. pneumophila*, we did not observe a clear requirement for the *C. burnetii* T4SS in the cytokine response. Instead, we observed a slight decrease in TNF and IL-6 production in response to T4SS-sufficient *C. burnetii* compared to a T4SS-mutant strain. While the difference was not robust, it suggests the

intriguing possibility that *C. burnetii* T4SS effectors inhibit cytokine production. Inflammasomes are another cytosolic immune response that are robustly activated and play a critical role in controlling a variety of pathogens, including *L. pneumophila* (Casson and Shin, 2013). Unlike *L. pneumophila*, *C. burnetii* does not possess flagellin (Seshadri et al., 2003), a potent activator of the NAIP5 inflammasome (Casson et al., 2013). It is unknown, however, if *C. burnetii* possesses other stimulators of inflammasome activation, as is the case for *L. pneumophila*. We found that while *C. burnetii* induces pro-IL-1 β production, it does not induce inflammasome activation. This could be because *C. burnetii* does not express PAMPs that are recognized by inflammasome-associated PRRs. For example, caspase-11 recognizes hexa- or penta-acylated LPS, but *C. burnetii* generates tetra-acylated LPS (Zamboni et al., 2004), which would not be expected to activate caspase-11 (Hagar et al., 2013). Alternatively, *C. burnetii* may encode T4SS effectors that actively inhibit inflammasome activation. It has been shown, however, that primary human alveolar macrophages produce mature IL-1 β in response to *C. burnetii* NMII, but not phase I *C. burnetii*, suggesting that immune sensing pathways in mouse and human cells may respond differently to NMII *C. burnetii* (Graham et al., 2013). Our findings that *C. burnetii* does not robustly induce inflammasome activation in mouse cells may provide some insight into the observation that *C. burnetii* does not elicit robust neutrophil recruitment during *in vivo* infection of mice (Elliott et al., 2013), as inflammasome-derived IL-1 is a critical regulator of neutrophil recruitment during pulmonary *L. pneumophila* infection (Barry et al., 2013; Casson et al., 2013; LeibundGut-Landmann et al., 2011; Mascarenhas et al., 2015). Our findings raise the intriguing possibility that *C. burnetii* has evolved strategies to evade or actively inhibit cytosolic innate immune detection.

Many intracellular bacteria induce cytosolic immunosurveillance pathways that lead to type I IFN expression, and this response can be both beneficial to the host by limiting intracellular bacterial replication, as is the case for *L. pneumophila* (Schiavoni et al., 2004), or detrimental to the host, as is the case for *M. tuberculosis* (Manzanillo et al., 2012), by promoting intracellular bacterial replication. It is unknown, however, if type I IFNs are induced or play a role in controlling *C. burnetii* infection. Our studies indicate that *C. burnetii* infection induces very low, but biologically active levels of type I IFNs. Furthermore, we found that BMDMs lacking the type I IFN receptor did not exhibit increased bacterial replication, suggesting that type I IFNs do not participate in restricting *C. burnetii* replication in B6 macrophages.

As our studies suggested a more prominent role for TLRs rather than cytosolic immune sensing pathways in responding to *C. burnetii*, we next examined the mechanistic basis underlying the link between TLR recognition and control of bacterial replication. Given that our data and other published studies demonstrated a critical role for TLR signaling in mediating production of proinflammatory cytokines during *C. burnetii* infection, we wondered whether cytokines play a role in restricting *C. burnetii* replication. Interestingly, BMDMs lacking only Trif did not have a defect in limiting intracellular *C. burnetii* replication despite having a defect in TNF production, whereas MyD88-deficient BMDMs had a defect in both TNF production and control of *C. burnetii* replication that resembles *Tlr2*^{-/-} BMDMs. We were able to uncover a role for Trif in limiting replication of *C. burnetii* only when we deleted both Trif and MyD88, which led to increased bacterial replication compared to deletion of MyD88 alone. These findings suggest that in addition to TNF production, TLR2 and MyD88 signaling are required for other antibacterial effector mechanisms that restrict *C. burnetii*.

In conclusion, our findings highlight the importance of TLR signaling in enabling B6 macrophages to restrict *C. burnetii* NMII. Our data also indicate that *C. burnetii* NMII T4SS activity does not robustly trigger several cytosolic immunosurveillance pathways that we examined, nor do these pathways play a major role in the control of intracellular *C. burnetii* replication in B6 macrophages. Our studies provide further insight into innate immune pathways used by macrophages to sense and restrict the replication of this poorly understood pathogen.

Acknowledgements

We thank Susan Ross for providing *Tlr2^{-/-}*, *Tlr4^{-/-}*, *Tlr2^{-/-}Tlr4^{-/-}*, and *Myd88^{-/-}* mice, Jeffrey Weiser for providing *Tlr2^{-/-}* mice, and Igor Brodsky for providing *Tlr2^{-/-}Tlr4^{-/-}* and *Myd88^{-/-}* *Trif^{-/-}* mouse bone marrow. We also thank Alan Copenhaver for technical advice and members of the Shin, Brodsky, and Roy laboratories for helpful discussions and advice. This work was supported in part by National Institutes of Health grants K99/R00AI087963 (S.S.), R01AI048770 (C.R.R.), T32AI055400 (L.B.), American Lung Association grant RG-268528-N (S.S.), University of Pennsylvania University Research Foundation (S.S.), Institute for Immunology Pilot Award (S.S.), and American Heart Association grant 13BGIA14780070 (S.S.).

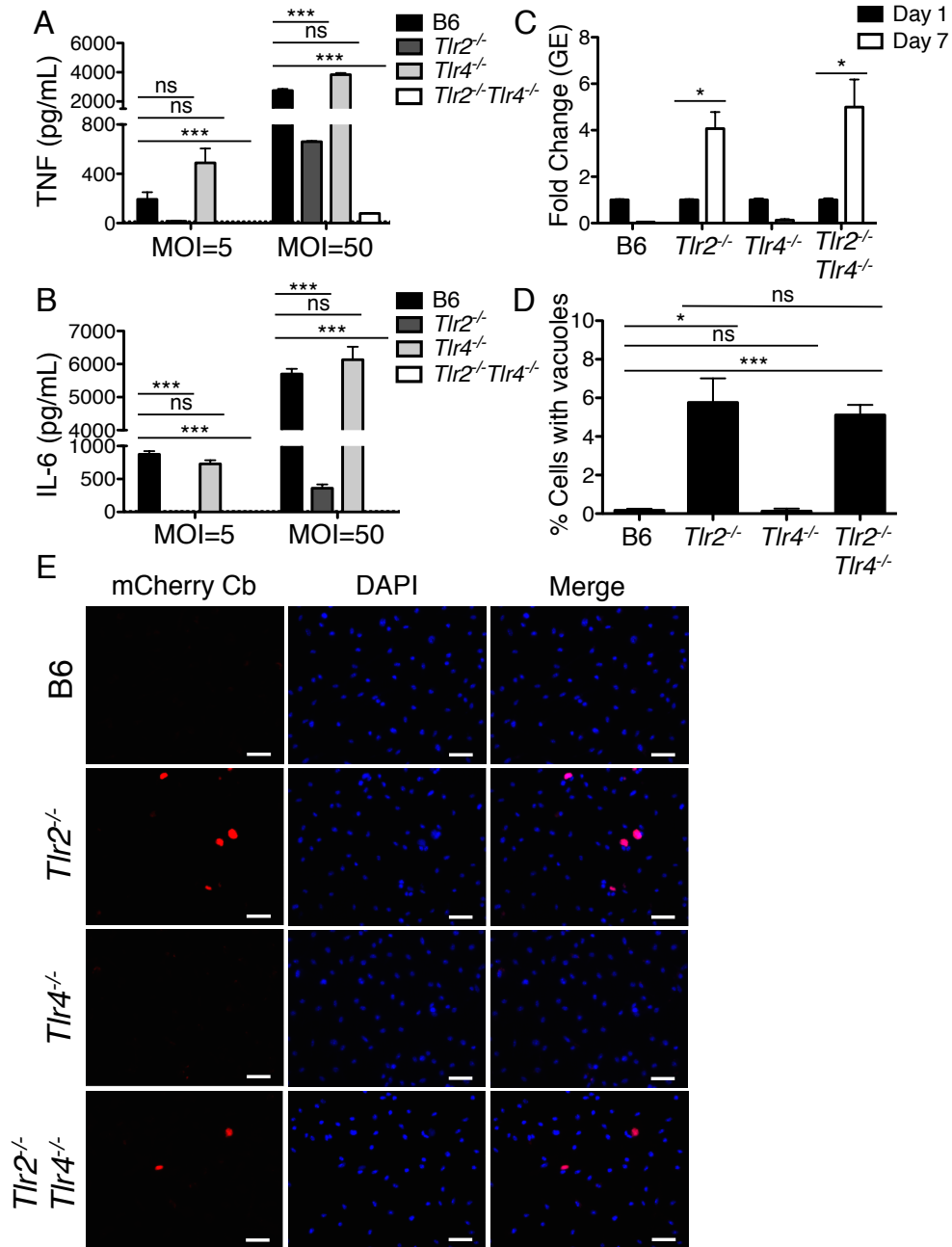


Figure 2.1 TLR2 and TLR4 mediate immune responses to *Coxiella burnetii* in mouse macrophages (A and B) C57BL/6, *Tlr2*^{-/-}, *Tlr4*^{-/-}, and *Tlr2*^{-/-}*Tlr4*^{-/-} bone marrow-derived macrophages (BMDMs) were infected with WT *C. burnetii* NMII at MOI=5 or 50 for 24 hours. Levels of TNF and IL-6 in the supernatants were measured by ELISA. Graphs show the mean \pm SEM of triplicate wells. Representative of three independent

experiments. (C) C57BL/6, *Tlr2*^{-/-}, *Tlr4*^{-/-}, and *Tlr2*^{-/-}*Tlr4*^{-/-} BMDMs were infected with WT *C. burnetii* NMII at MOI=100. At days 1 and 7 post-infection, bacterial uptake and replication was measured as genomic equivalents (GEs) by qPCR. Graphs show the fold change in GEs on day 7 relative to GEs on day 1 \pm SEM of triplicate wells. (D) 7 days post-infection, BMDMs of the indicated genotypes infected with mCherry-expressing WT *C. burnetii* NMII at MOI=100 were fixed, stained with DAPI, and examined by fluorescence microscopy. The number of mCherry-expressing *C. burnetii*-containing vacuoles was enumerated and calculated as a percentage of the total cell number. Graphs show the mean percentage of cells containing *C. burnetii* vacuoles \pm SEM of triplicate coverslips. At least 300 cells were counted per coverslip. Representative of two independent experiments. (E) Representative fluorescence micrographs of mouse BMDMs of the indicated genotypes infected with mCherry-expressing *C. burnetii* (Cb) at MOI=100 and fixed and stained with DAPI on day 7 post-infection. Images were taken at 40X magnification. Scale bars represent 25 μ M. *p<0.05, **p<0.01, ***p<0.001, ns=no significance.

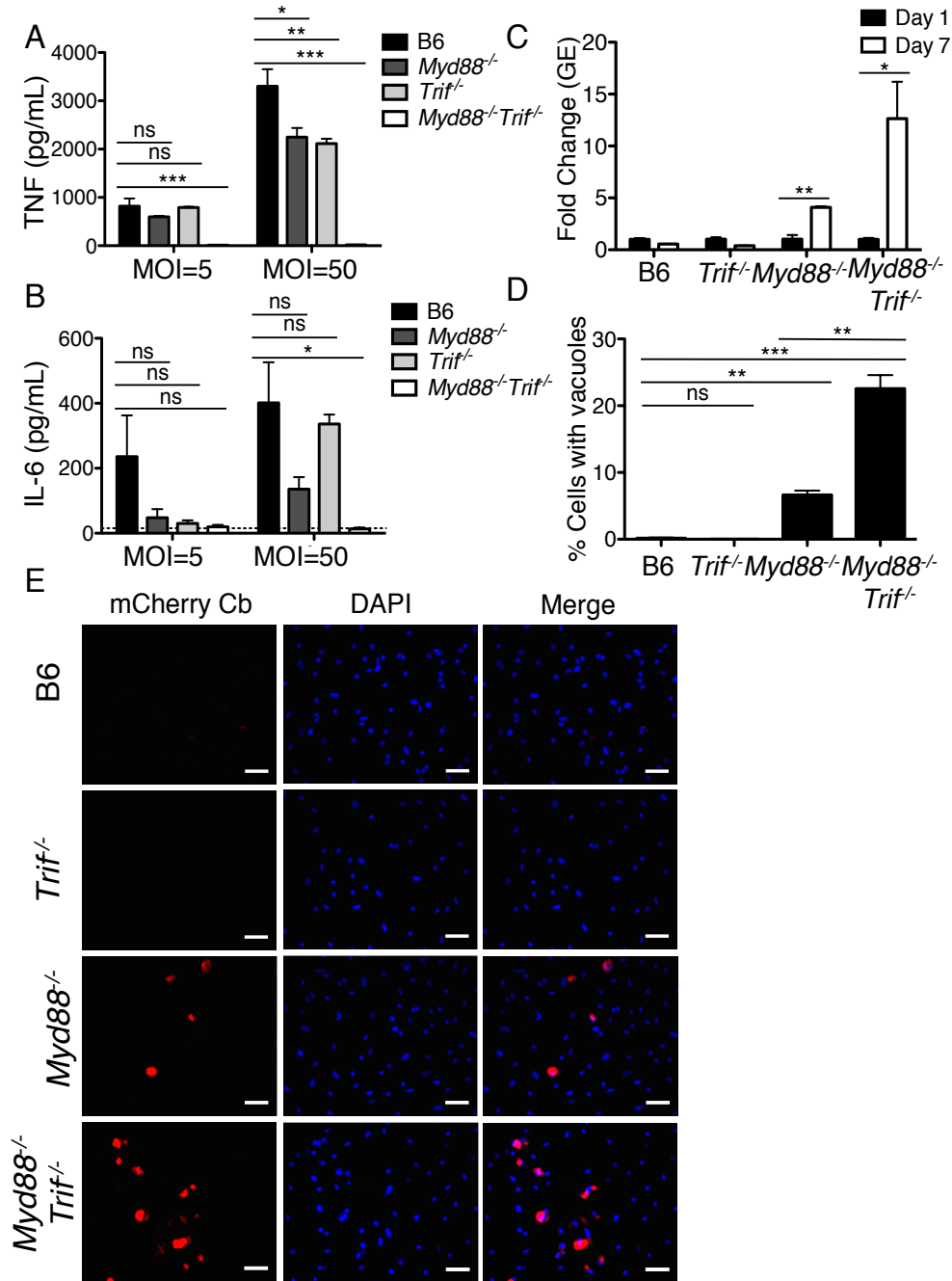


Figure 2.2 Myd88 and Trif are required for cytokine production and the restriction of intracellular growth (A and B) C57BL/6, *Trif*^{-/-}, *Myd88*^{-/-}, and *Myd88*^{-/-}*Trif*^{-/-} BMDMs were infected with WT *C. burnetii* NMII at MOIs= 5 and 50 for 24 hours. Levels of TNF and IL-6 in the supernatants were measured by ELISA. Graphs show the mean \pm SEM

of triplicate wells. Representative of two independent experiments (C) C57BL/6, *Trif*^{-/-}, *Myd88*^{-/-}, and *Myd88*^{-/-}*Trif*^{-/-} BMDMs were infected with WT *C. burnetii* NMII at MOI=100. At days 1 and 7 post-infection, bacterial uptake and replication was measured as genomic equivalents (GEs) by qPCR. Graphs show the fold change in GEs relative to the GEs measured on day 1 ± SEM of triplicate wells. (D)) 7 days post-infection, BMDMs of the indicated genotypes infected with mCherry-expressing WT *C. burnetii* NMII at MOI=100 were fixed, stained with DAPI, and examined by fluorescence microscopy. The number of *C. burnetii*-containing vacuoles was enumerated and calculated as a percentage of the total cell number on day 7 post-infection. Graphs show the mean percentage of cells containing *C. burnetii* vacuoles ± SEM of triplicate coverslips. At least 300 cells were counted per coverslip. Representative of two independent experiments. (E) Representative fluorescence micrographs of BMDMs of the indicated genotypes infected with mCherry-expressing *C. burnetii* (Cb) at MOI=100 and fixed and stained with DAPI on day 7 post-infection. Images were taken at 40X magnification. Scale bars represent 25 μM. *p<0.05, **p<0.01, ***p<0.001, ns=no significance.

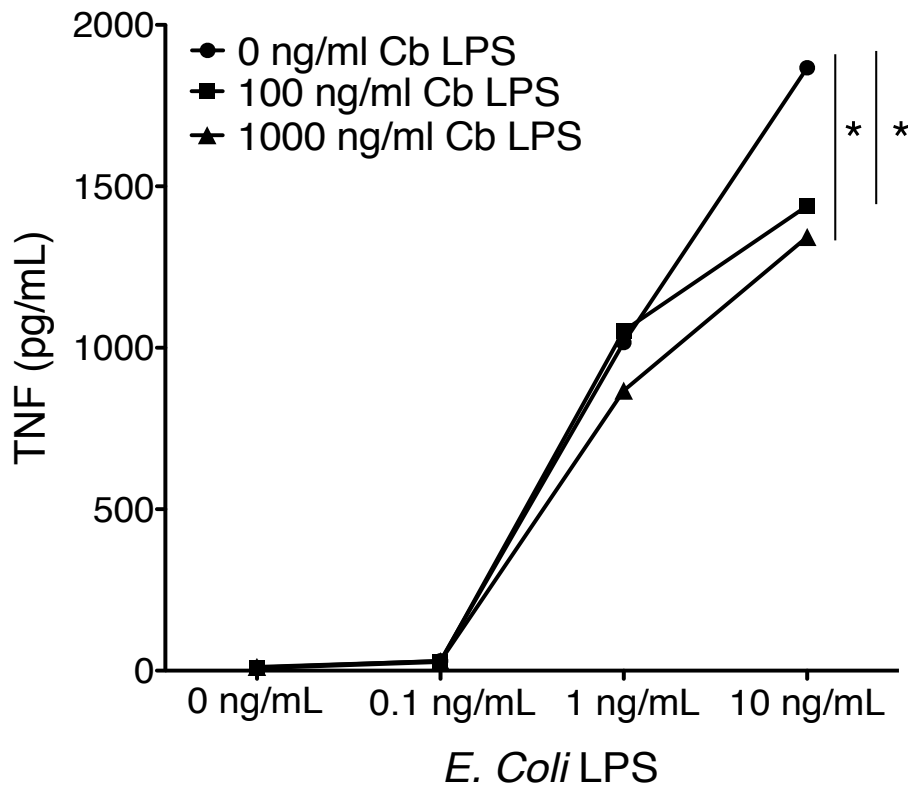


Figure 2.3 Purified *C. burnetii* LPS does not induce TNF production. C57BL/6 BMDMs were pre-treated for various concentrations of purified *C. burnetii* Nine Mile phase II LPS (Cb) for 30 min then treated with various concentrations of *E. coli* LPS for 24 hours. TNF was measured by ELISA. Graphs represent means of triplicate wells. Experiment is representative of two experiments. *p<0.05

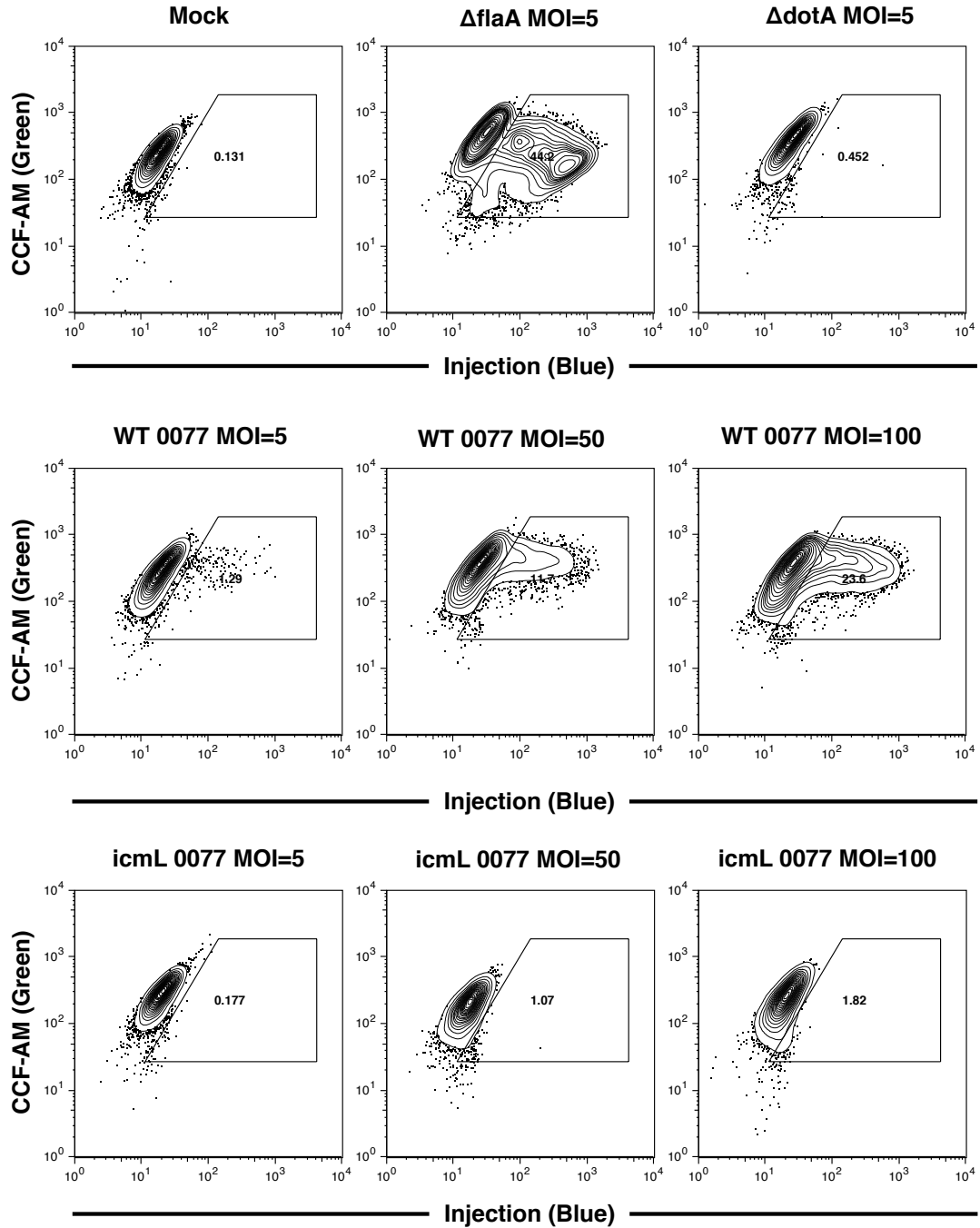


Figure 2.4 *C. burnetii*'s T4SS is capable of injecting effectors into C57BL/6 BMDMs

C57BL/6 BMDMs were treated with RPMI or infected, and $\Delta flaA$ and $\Delta dotA$ strains expressing BlaM-RaIF MOI=5, or with WT and *icmL*::Tn expressing BlaM-CBU_0077 at MOI= 5, 50 and 100. 16 hours post infection Cells were then loaded with CCF4-AM and

analyzed by flow cytometry. Cells positive for cleaved CCF4-AM (blue) fluorescence were gated and the percentage injection is denoted in each gate. Experiment is representative of two experiments.

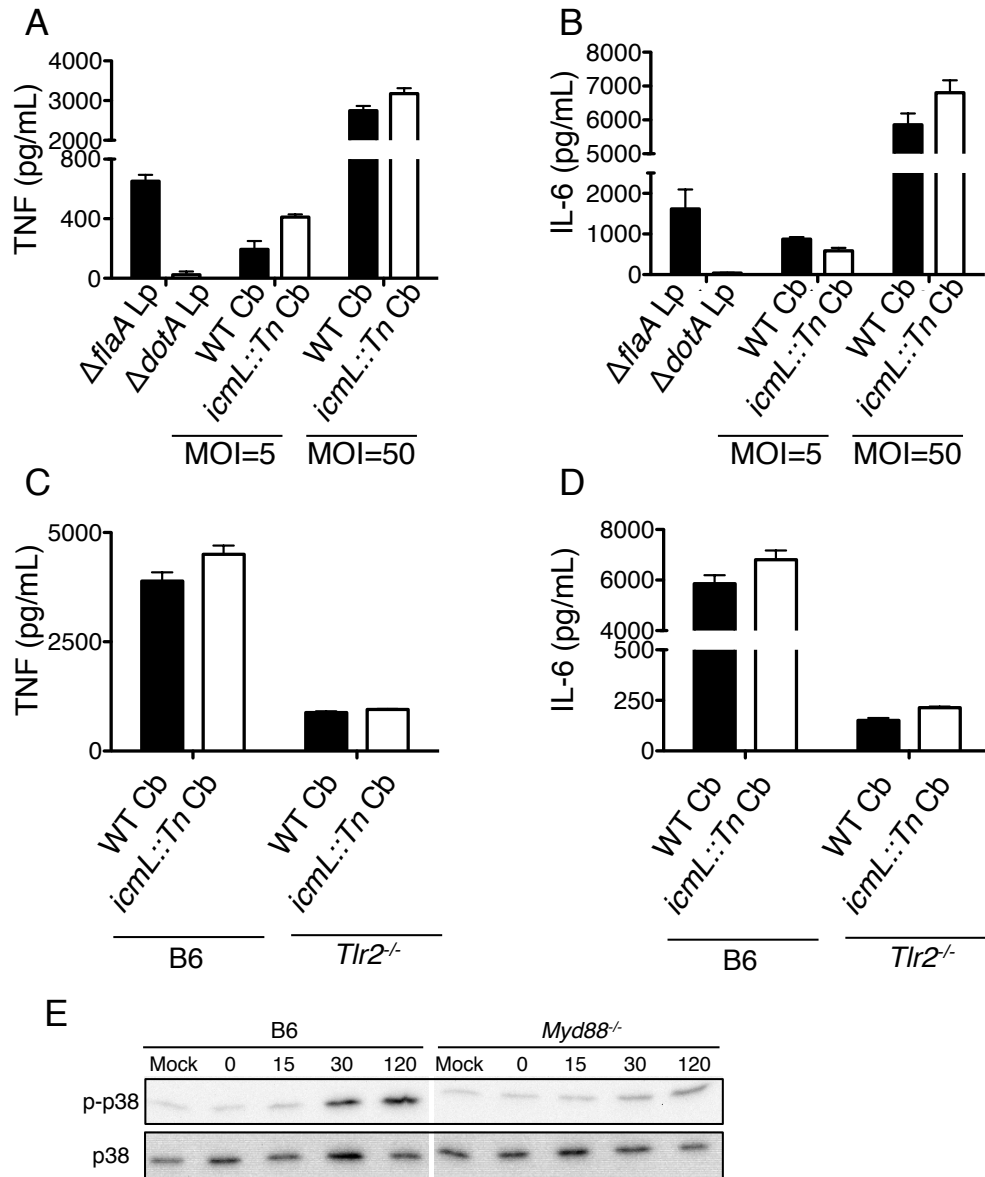


Figure 2.5 *C. burnetii* T4SS expression does not lead to enhanced cytokine

production or early p38 MAPK phosphorylation. (A and B) C57BL/6 BMDMs were infected with *L. pneumophila* (Lp) $\Delta flaA$ or $\Delta dotA$ at MOI=5, or WT *C. burnetii* (Cb) or *icmL::Tn* at MOI=5 or 50 for 24 hours. Levels of (A) TNF and (B) IL-6 in the supernatants were measured by ELISA. Graphs show the mean \pm SEM of triplicate wells. Representative of three independent experiments.

(C and D) C57BL/6 BMDMs and *Tlr2*^{-/-} were infected with WT *C. burnetii* (Cb) or *icmL::Tn* at MOI= 50 for 24 hours. Levels of (C)

TNF and (D) IL-6 in the supernatants were measured by ELISA. Graphs show the mean \pm SEM of triplicate wells. Representative of two independent experiments (E)

Immunoblot analysis of phosphorylated and total p38 MAPK in cell lysates from C57BL/6 and *Myd88*^{-/-} BMDMs that were mock-infected or infected with WT *C. burnetii* NMII at MOI=50 for 0, 15, 30, or 120 minutes. Representative of two independent experiments.

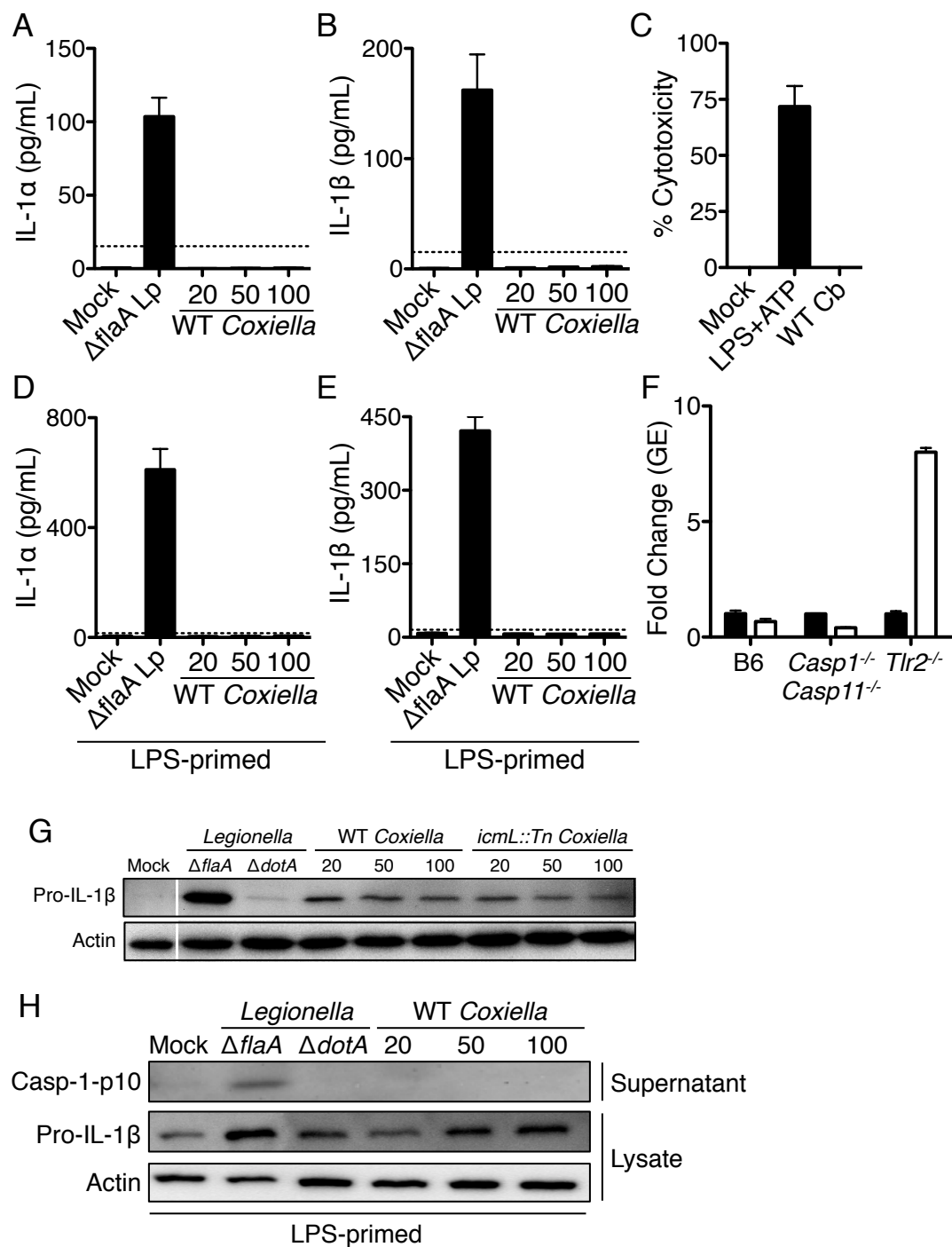


Figure 2.6 *Coxiella burnetii* infection does not induce detectable inflammasome activation. (A and B) Unprimed C57BL/6 BMDMs were mock-infected, infected with *L. pneumophila* $\Delta fliA$ at MOI=5, or infected with WT *C. burnetii* NMII at MOI= 20, 50, or 100 for 24 hours. Levels of (A) IL-1α and (B) IL-1β were measured in the supernatants

by ELISA. Graphs show the mean \pm SEM of triplicate wells. Representative of three independent experiments. (C) C57BL/6 BMDMs were treated with 0.5 ug/mL LPS for 4 hours followed by 2.5mM ATP for one hour or infected with WT *C. burnetii* NMII at MOI=50 for 24 hours. % cytotoxicity was measured via LDH release. Graph shows the mean \pm SEM of triplicate wells. (D and E) C57BL/6 BMDMs were first primed with 0.5 ug/mL LPS for 4 hours and then either mock-infected, infected with *L. pneumophila* $\Delta flaA$ at MOI=5, or infected with WT *C. burnetii* at MOIs=20, 50, 100 for 24 hours. Levels of (D) IL-1 α and (E) IL-1 β were measured in the supernatants by ELISA. Graphs show the mean \pm SEM of triplicate wells. Representative of two independent experiments. (F) C57BL/6, *Tlr2*^{-/-}, and *Casp1*^{-/-}*Casp11*^{-/-} BMDMs were infected with WT NMII *C. burnetii* at MOI=100. At days 1 and 7 post-infection, bacterial uptake and replication was measured as genomic equivalents (GEs) by qPCR. Graphs show the fold change in GEs on day 7 relative to GEs on day 1 \pm SEM of triplicate wells. (G) Immunoblot analysis of pro-IL-1 β and actin in cell lysates from unprimed C57BL/6 BMDMs that were mock-infected or infected with *L. pneumophila* $\Delta flaA$ or $\Delta dotA$ at MOI=5, or WT *C. burnetii* or *icmL::Tn* at MOI=20, 50, or 100 for 24 hours. Representative of two independent experiments. (H) Immunoblot analysis of processed caspase-1 (casp-1 p10) in the supernatant of primed C57BL/6 BMDMs and pro-IL-1 β and actin in the cell lysate. Representative of two independent experiments.

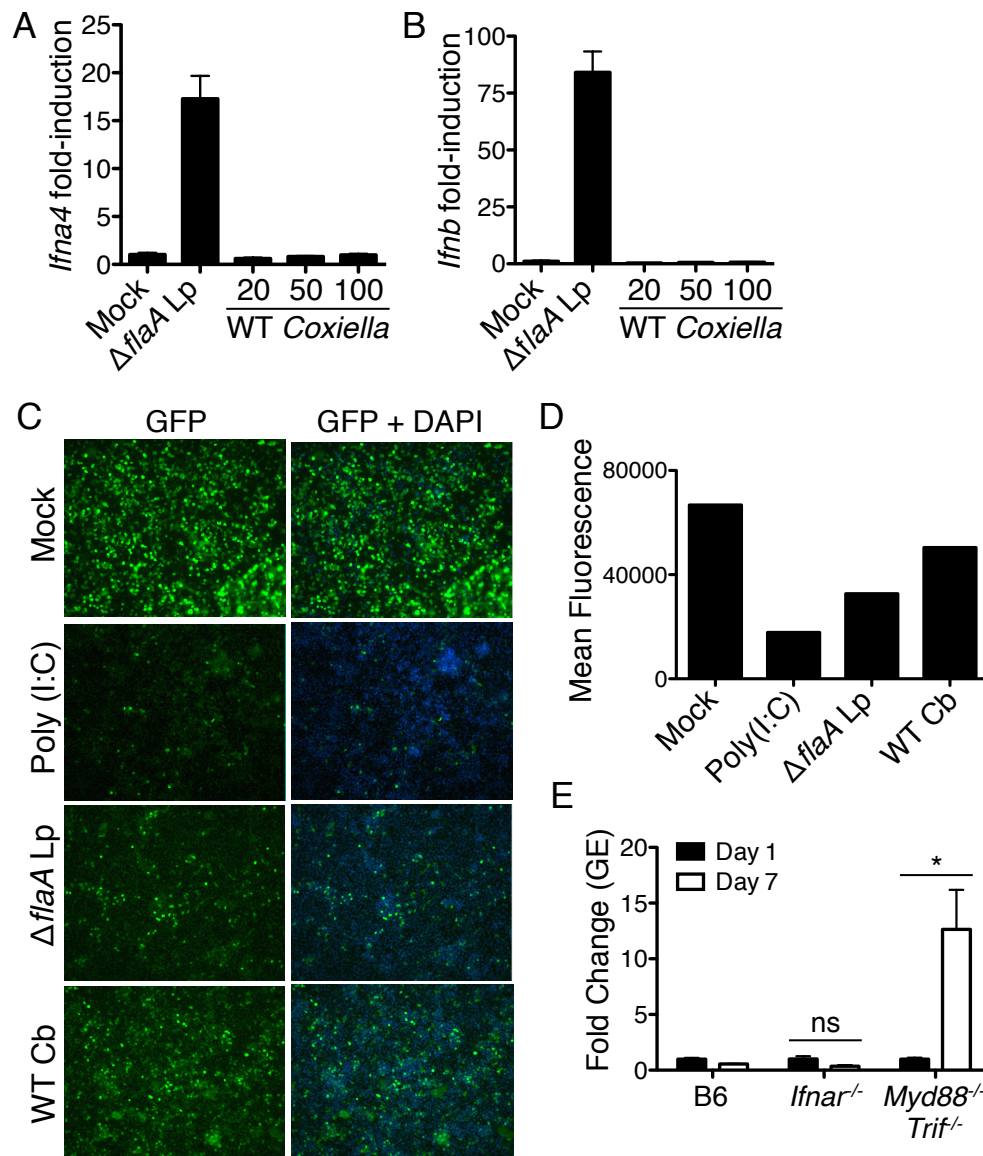


Figure 2.7 *Coxiella burnetii* infection does not induce robust type I interferon production and intracellular replication is not restricted by type I interferon signaling. (A and B) C57BL/6 BMDMs were mock-infected, infected with *L.*

pneumophila Δ *flaA* at MOI=10, or infected with WT *C. burnetii* NMII at MOI=10, 50, or 100 for 16 hours. Fold-induction of *Ifna4* and *Ifnb* mRNAs were measured via qRT-PCR.

Graphs show the mean \pm SEM of triplicate wells. Representative of two independent

experiments. (C) Supernatants from C57BL/6 BMDMs that were mock-infected, infected

with *L. pneumophila* $\Delta flaA$ at MOI=10, or infected with WT *C. burnetii* NMII at MOI=50 for 16 hours were incubated with L2 mouse fibroblasts for 24 h. L2 cells were then infected with NDV-GFP at an MOI=1. As a positive control, L2 cells were treated with supernatants from C57BL/6 BMDMs stimulated with poly (I:C) for 16 hours. At 24 hours post-infection, cells were fixed, stained with DAPI, and examined for levels of NDV-GFP replication by fluorescence microscopy. Shown are representative fluorescence micrographs. (D) Graphs of mean GFP fluorescence from each sample quantified with ImageJ software. Representative of two independent experiments. (E) C57BL/6, *Ifnar*^{-/-}, or *Myd88*^{-/-} *Trif*^{-/-} BMDMs were infected with WT *C. burnetii* NMII at MOI=100. *C. burnetii* GEs were measured qPCR on days 1 and 7 post-infection. Graphs show the fold change in GEs relative to day 1 \pm SEM of triplicate wells. Representative of two independent experiments.

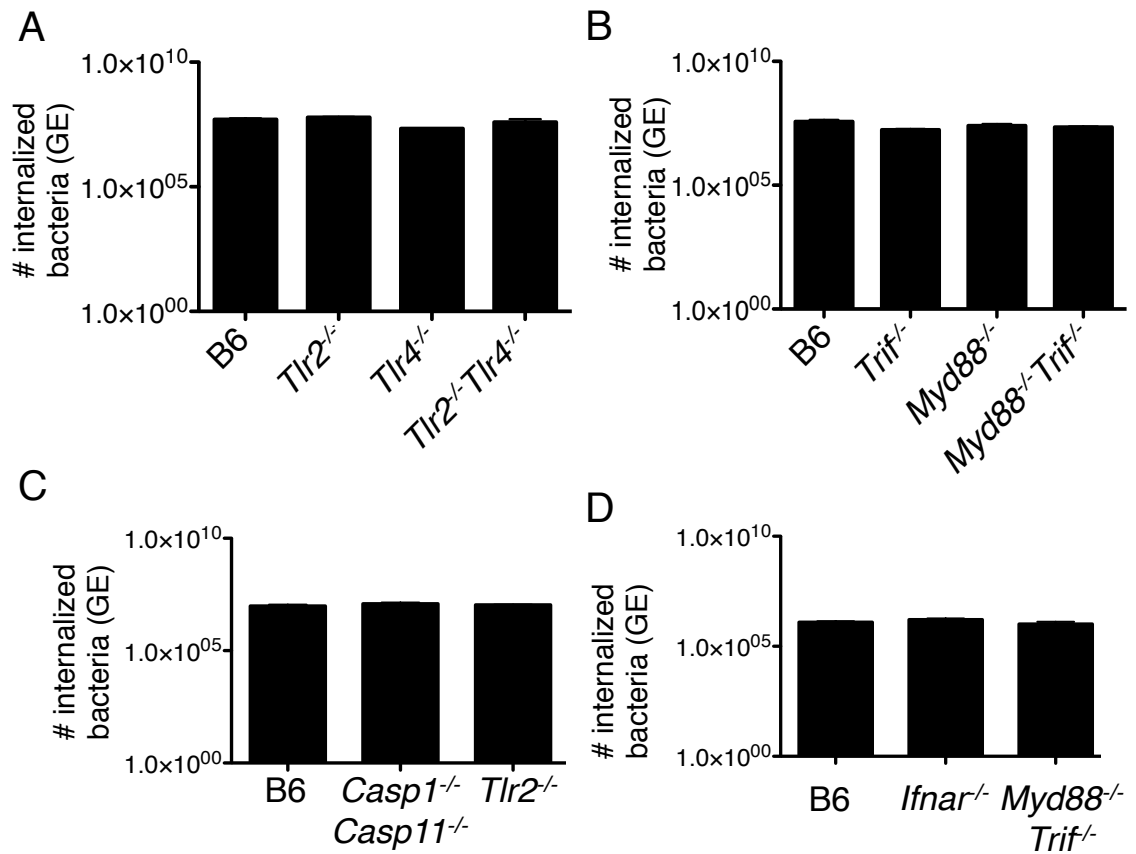


Figure 2.8. Levels of internalized WT *Coxiella burnetii* are similar for different genotypes of bone marrow-derived macrophages at 24 hours post-infection. 24 hours post-infection, the number of internalized WT *C. burnetii* NMII genomic equivalents (GEs) were measured by qPCR for (A) C57BL/6, *Tlr2*^{-/-}, *Tlr4*^{-/-}, and *Tlr2*^{-/-}*Tlr4*^{-/-} BMDMs presented in Figure 2.1C, (B) C57BL/6, *Trif*^{-/-}, *Myd88*^{-/-}, and *Myd88*^{-/-}*Trif*^{-/-} BMDMs presented in Figure 2.2C, (C) C57BL/6, *Tlr2*^{-/-}, and *Casp1*^{-/-}*Casp11*^{-/-} BMDMs presented in Figure 2.6F, and (D) C57BL/6, *Ifnar*^{-/-}, *Myd88*^{-/-}*Trif*^{-/-} BMDMs presented in Figure 2.7E.

CHAPTER 3:

TNF-mediated control of *C. burnetii*

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Abstract

Pathogen activation of surface and cytosolic sensors triggers a variety of innate immune responses. Our studies described in the previous chapter have shown that *Coxiella burnetii* does not robustly activate cytosolic production of pro-inflammatory cytokines, nor does it activate the inflammasome. Instead, the innate immune response to *C. burnetii* relied largely on TLR-mediated detection, with TLR2 playing the dominant role in the production of pro-inflammatory cytokines and cell-intrinsic control of *C. burnetii* replication *in vitro*. Building upon these observations, we were further able to implicate TNF as a mediator of cell-intrinsic control of bacterial replication, as TNF-deficient macrophages were permissive for *C. burnetii* replication. Furthermore, we found that soluble TNF is able to restrict *C. burnetii* replication in *Tlr2*^{-/-} macrophages. To elucidate the mechanism by which TNF mediates cell-intrinsic control of *C. burnetii* replication, we tested three possible TNF-mediated bacterial restriction mechanisms: cell death, production of ROS and RNS, and Guanylate Binding Proteins (GBPs). We found that *C. burnetii* does not induce robust cell death, indicating that TNF-induced extrinsic apoptosis and programmed necrosis are not major control mechanisms. We found *C. burnetii* induces RNS in a TNF-dependent manner. *C. burnetii* also induces whole cell ROS, which plays a role in restricting bacterial replication. GBP1 and GBP2 are induced by *C. burnetii* infection, but we did not find a role for these or three additional GBPs in bacterial restriction. Overall, these findings implicate the cytokine TNF, which is induced by *C. burnetii* in a TLR-dependent manner, as mediating restriction of *C. burnetii* replication and further examines possible mechanisms of TNF-mediated restriction.

Introduction

The downstream consequences of cell surface and cytosolic immune detection are critical for successful pathogen clearance. Following pathogen activation of cell surface and cytosolic immune sensors, immune cells release a set of cytokines and chemokines. These cytokines and chemokines, in turn, can signal to other cells to induce an cell-extrinsic immune response or signal back to the compromised cell to induce an cell-intrinsic immune response (Kawai and Akira, 2009).

Tumor necrosis factor (TNF) is a well-studied cytokine that is expressed in response to a wide array of pathogens (Akira, 2003). In the context of bacterial infection, PAMPs stimulate TLR and cytosolic immune receptors to activate the NF- κ B and MAPK signaling pathways to induce the transcription of TNF (Akira, 2003), with macrophages thought to be a major source of TNF (Wajant et al., 2003). TNF is expressed as a type II transmembrane protein that arranges into homotrimers (Kriegler et al., 1988; Tang et al., 1996). The soluble form is released via proteolytic cleavage by the metalloprotease TNF alpha-converting enzyme (TACE) (Black et al., 1997). Two TNF receptor (TNF-R) family members, TNF-R1 and TNF-R2, bind soluble and membrane-bound TNF and induce a variety of cell-intrinsic and cell-extrinsic immune responses (Locksley et al., 2001). While membrane-bound TNF strongly binds both TNF-R1 and TNF-R2 receptors, soluble TNF preferentially signals through TNF-R1 (Grell et al., 1995; Wallach et al., 1991). TNF-R1 is ubiquitously expressed on most cell types, and TNF-R2 is only expressed on immune cells (Locksley et al., 2001). The activity of TNF is broad and it has been found to play a major role in pathogen and tumor clearance (Wajant et al., 2003). Overproduction or prolonged production of TNF can drive pathological inflammation leading to septic shock or a number of autoimmune pathologies including rheumatoid arthritis and inflammatory

bowel disease (Blam et al., 2001; Taylor et al., 2000). Several anti-TNF therapies have been developed to decrease TNF-dependent autoimmune inflammation (Taylor, 2001). These therapies have proven effective at alleviating the symptoms of many autoimmune diseases, but have also been linked to increased susceptibility to certain pathogens, notably *M. tuberculosis* and *L. pneumophila* (Lanternier et al., 2013; Wagner et al., 2002). This recent phenomenon highlights TNF's importance in protection against intracellular bacteria.

TNF induces a host of cell-intrinsic immune responses that aid in the clearance of pathogens, including but not limited to: apoptotic and necroptotic cell death (Schulze-Osthoff et al., 1998; Tartaglia et al., 1993); induction of reactive oxygen (ROS) and reactive nitrogen (RNS) species (Chakravorty and Hensel, 2003; Han et al., 2009); and the induction of guanylate binding proteins (GBPs) (Vestal and Jeyaratnam, 2011). TNF-induced cell death occurs in two forms: caspase-8 mediated extrinsic apoptosis and caspase-independent programmed necrosis. TNF binding alone induces pro-survival cascades which upregulate anti-apoptotic proteins, such as c-FLIP (Wajant et al., 2003). c-FLIP shares sequence homology with the death caspase, caspase-8, but lacks a catalytic domain. It binds caspase-8 to prevent caspase-8::caspase-8 homodimerization and subsequent activation (Micheau and Tschopp, 2003; Schulze-Osthoff et al., 1998). When host protein synthesis is disrupted and c-FLIP is not upregulated, TNF signaling induces caspase-8 activation. Active caspase-8 leads to the processing and activation of the terminal death caspase, caspase-3, which initiates DNA fragmentation, membrane blebbing, and cell death. Caspase-8 can activate caspase-3 via direct processing or through the mitochondrial pathway, whereby cytochrome C is released from permeabilized

mitochondria activates caspase-9, which then activates caspase-3 to initiate cell death (Micheau and Tschopp, 2003).

Programmed necrosis, or necroptosis, occurs as a backup when extrinsic apoptosis is inhibited by viral inhibitors such as vICA and CrmA (Ray and Pickup, 1996; Skaletskaya et al., 2001). Necroptosis can be induced by TNF, Toll-like receptors (TLR3 and TLR4), or DNA sensors (DAI) (Vandenabeele et al., 2010). When caspase-8 activation is inhibited, TNF bound to TNFR-1 is internalized and initiates the assembly of a RIPK1-RIPK3 complex, termed the necrosome. In this complex, both RIPK1 and RIPK3 are phosphorylated and initiate a series of necroptotic events that lead to cell death and the release of alarmins, such as IL-1 α , IL-33, and Danger Associated Molecular Patterns (DAMPs), such as heat shock proteins and ATP (Vandenabeele et al., 2010). DAMPs have the potential to trigger surface and cytosolic immune receptors as well. Both apoptosis and necroptosis are thought to control intracellular pathogens by eliminating their replicative niche.

TNF also has the potential to induce ROS and RNS, which have a broad spectrum of anti-microbial activities (Wajant et al., 2003). ROS is generated by the constitutively expressed NADPH phagocytic oxidase (Phox), while RNS is generated by inducible nitric oxide synthase (iNOS) (Bylund et al., 2010; Nathan and Shiloh, 2000). The formation of the phox complex produces oxygen radicals via electron transfer, which in turn spontaneously generate hydrogen peroxide intermediates. Highly toxic hydroxyl radicals and hypochlorous acid are further generated via the Fenton reaction or by cellular myeloperoxidase (MPO) (Imlay and Linn, 1988; Imlay, 2008; Tlili et al., 2011). These reactive oxygen intermediates likely target bacterial DNA and can also act as signaling molecules to induce production of inflammatory cytokines and cell death. RNS

production requires the *de novo* synthesis of inducible nitric oxide synthase (iNOS), which contains a N-terminal oxygenase and a C-terminal reductase. Upon expression, iNOS forms homodimers and localizes to the phagosome in an actin-dependent manner to synthesize nitric oxide radicals from L-arginine substrate (Albakri and Stuehr, 1996; Baek et al., 1993; Davis et al., 2007). Nitrogen radicals target a variety of bacterial components, including bacterial DNA, proteins, and lipids. Bacterial death and replication inhibition results primarily via disruption of components of the bacterial respiration pathway (Nathan and Shiloh, 2000).

Guanylate binding proteins (GBPs) are additional cell-intrinsic immune effectors that can be activated by TNF (Martens and Howard, 2006). Mice encode 11 GBPs (mGBP-1 to mGBP11), while humans encode 7 GBPs (hGBP-1 to hGBP-7) (Olszewski et al., 2006). GBPs are classically thought to be induced by Type I and Type II interferons, but select GBPs have been shown to be induced by IL-1 α , IL-1 β and TNF (Vestal and Jeyaratnam, 2011). Upregulated GBPs are targeted to cellular membranes by isoprenylation, which involves the addition of a C-15 farnesyl or C-20 geranylgeranyl lipid moieties to the cysteine residue in the conserved, C-terminal CaaX sequence (Vestal and Jeyaratnam, 2011). Both mice and human GBPs have been implicated in the control of intracellular pathogens (Nguyen et al., 2002). For example, hGBP-1 and hGBP-2 inhibit replication of *Chlamydia spp* (Tietzel et al., 2009); mGBP-2 controls replication of *Toxoplasma gondii* (Degrandi et al., 2013); and mGBP-5 promotes *Salmonella* Typhimurium-induced pyroptosis (Pilla et al., 2014).

C. burnetii has evolved strategies to subvert some of these pathways. It is well-characterized that *C. burnetii* inhibits artificially induced intrinsic apoptosis through the T4SS effectors AnkG, CaeA, and CaeB (Klingenberg et al., 2013; Lührmann et al.,

2010). To date, only one study has offered insight into TNF-mediated extrinsic apoptosis. In that study, THP-1 cells infected with *C. burnetii* were treated with recombinant TNF and cyclohexamide and reduced cleavage of PARP, a terminal marker of apoptosis, was observed (Voth et al., 2007). This study, however, did not link this inhibition to a functional T4SS, nor did it show *C. burnetii* has the potential to induce caspase-8 mediated apoptosis occurs. Another study also found that at a relatively early time point, 24 hours post-infection, *C. burnetii* induces a caspase-independent form of cell death that is partially mediated by TNF (Zhang et al., 2012). These studies offer the possibility of both apoptotic and necroptotic cell death as mechanisms to control *C. burnetii* or pathways that are evaded by *C. burnetii*.

Studies on the role and significance that ROS and RNS play in the restriction of *C. burnetii* have varying conclusions. Some studies concluded that *C. burnetii* does not elicit a detectable oxidative burst in monocytes and therefore does not produce either ROS or RNS in human neutrophils or macrophages (Akporiaye et al., 1990; Dellacasagrande et al., 1999). Moreover, *C. burnetii* growth did not differ between monocytes from healthy human donors and those with chronic granulomatous disease, which lack a functional NADPH (Akporiaye et al., 1990). Other studies with macrophages treated with exogenous IFN γ showed *C. burnetii* infection did induce both ROS and RNS, which were both shown to play a role in growth restriction upon stimulation in the presence of exogenous IFN γ (Brennan et al., 2004; Zamboni and Rabinovitch, 2003). Like many pathogens, *C. burnetii* has evolved strategies to combat ROS and RNS stress. It was shown that phagocytosis of *C. burnetii* by neutrophils prevented assembly of the NADPH oxidase on the phagosomal membrane via an unknown mechanism, reducing whole cell levels of ROS (Siemsen et al., 2009). It was also demonstrated that

C. burnetii secretes an acid phosphatase into the vacuole that reduces neutrophil ROS (Hill and Samuel, 2011). This *C. burnetii* enzyme may prevent the phosphorylation of an NADPH component. However, the role of ROS and RNS in the context of *C. burnetii* infection of macrophages and endogenously produced TNF remain poorly understood.

The role of GBPs in *C. burnetii* infection have not been studied. Because *C. burnetii* forms a large vacuole that acquires membrane from a variety of sources and fusion events, it is likely that GBPs are recruited to the vacuole in some capacity. Whether infection with *C. burnetii* induces the expression of these GBPs or if these proteins promote or inhibit bacterial replication is unknown.

In this study, we found a role for soluble TNF in the cell-intrinsic inhibition of *C. burnetii* Nine Mile phase II replication within B6 macrophages. We previously showed that *C. burnetii* does not induce cytosolic-mediated immune responses, nor Type I interferon. We did find a role for TLR-dependent restriction mediated by both adaptors Myd88 and Trif and implicated both TLR2 and TLR4 in the production of TNF. Building upon this, we found TNF plays a critical role in *in vitro* control of *C. burnetii* infection. We then analyzed three possible cell-intrinsic effector pathways by which TNF could be restricting bacterial replication: cell death, ROS and RNS, and GBP induction.

Materials and Methods

Bacteria Strains

All experiments in this study used *Coxiella burnetii* Nine Mile Phase II (clone 4; RSA 439). For *C. burnetii* infections, acidified citrate cysteine medium (ACCM-2) was inoculated with WT *C. burnetii* (Omsland et al., 2013) or WT *C. burnetii* expressing mCherry (Beare et al., 2009). *C. burnetii* was grown at 37°C in 5% CO₂ and 2.5% O₂ for

6 days to late log phase ($\sim 1.0 \times 10^9$ bacteria/mL) (Omsland et al., 2013). One day after inoculation, chloramphenicol (2.5 $\mu\text{g/mL}$) was added to mCherry cultures. To quantify *C. burnetii* genome equivalents for infection of macrophages, *C. burnetii* genomic DNA was isolated with the illustraTM bacteria genomicPrep Mini Spin Kit (GE Healthcare), and genomic equivalents were measured by quantitative PCR of the *C. burnetii* *dotA* gene using SYBR Green, the CFX96 qPCR machine (Bio-Rad Laboratories), and the following primers: *dotA* 5' (GCGCAATACGCTCAATCACA), *dotA* 3' (CCATGGCCCCAATTCTCT).

Mammalian Cell Culture

Bone marrow was isolated from the femurs, tibia, and humeri of C57BL/6 (Jackson), *Tlr2*^{-/-}, *Tnf*^{-/-} (Pasparakis et al., 1996), *GBP*^{Chrm3-/-} (Yamamoto et al., 2003), and *gp91phox*^{-/-} (Pollock et al., 1995) mice. Bone marrow cells were differentiated into macrophages for 7-8 days in RPMI containing 30% L929 cell supernatant and 20% FBS at 37°C in a humidified incubator (Casson et al., 2013). Macrophages were lifted with ice-cold PBS + 2 mM EDTA and replated one day prior to infection in RPMI containing 15% L929 cell supernatant and 10% FBS. Macrophages were plated into 96, 48 or 24 well plates at 1.0×10^5 , 1.5×10^5 or 2.0×10^5 cells per well, respectively. *C. burnetii* were washed once with PBS prior to infection of macrophages, and macrophages were infected with various MOIs of *C. burnetii*. *C. burnetii* were washed once with PBS prior to infection of macrophages. After infection, cells were spun at 1200 RPM for 5 minutes prior to incubation at 37°C. At designated timepoints, macrophage supernatants, whole cell lysates, or mRNA were collected.

Measuring Intracellular *Coxiella burnetii* Replication

Measuring intracellular replication of *C. burnetii* in C57BL/6, *Tlr2*^{-/-}, *Tnf*^{-/-}, *GBP^{Chrm3}*^{-/-}, and *gp91phox*^{-/-} BMDMs was performed as follows. Macrophages were infected with *C. burnetii* Nine Mile Phase II expressing mCherry at MOI=100 in 24 well plates. 24 hours post-infection, cells were washed 3 times with PBS to remove extracellular bacteria and fresh media was added to the cells every 2 days. At designated timepoints post-infection, the extracellular media was collected and the adherent macrophages were lysed with 1mL sterile dH₂O. The lysed samples were then combined with the extracellular media from the same well. Bacterial genomic DNA was purified and *C. burnetii* genomic equivalents (GE) were measured via qPCR using primers specific for the *C. burnetii* *dotA* gene. The fold change in bacterial GEs was calculated as a ratio of the number of GEs on a given day to the number of GEs on day 1. For experiments examining the role of TNF in restricting *C. burnetii* growth, 10ng/mL recombinant mouse TNF (Biolegend) or supernatants from B6 macrophages infected with *C. burnetii* for 24 hours were added to *C. burnetii*-infected macrophages on day 1 and day 5 post-infection.

Enumeration of *Coxiella burnetii*-containing vacuoles by microscopy

2.0 x 10⁵ cells were plated onto a glass coverslip per well in triplicate in 24 well plates for each condition. Seven days post-infection, cells were washed with PBS, stained with DAPI, and fixed with 4% paraformaldehyde. Images were taken with a Nikon Eclipse 2000E-U epifluorescence microscope and images were acquired with NIS Elements B4 4.10.01 software. Large vacuoles containing mCherry-expressing *C. burnetii* were enumerated as a percentage of total DAPI-positive cells. For each coverslip, greater than 300 cells were counted.

TUNEL Assay

2.0×10^5 cells were plated onto a glass coverslip per well in triplicate in 24 well plates for each condition. Cells were either mock infected with RPMI, treated with 1000 μ M staurosporine for 4 hours, or for infected with WT *C. burnetii* at MOI=100 for 24 hours. Post-treatment cells were washed with PBS and fixed with 4% paraformaldehyde. Cells were incubated with TUNEL enzyme and TUNEL dye in a humidified incubator for sixty minutes. Coverslips were mounted with Prolong-gold with DAPI. Images were taken with a Nikon Eclipse 2000E-U epifluorescence microscope and images were acquired with NIS Elements B4 4.10.01 software. TUNEL positive cells were enumerated as a percentage of total DAPI-positive cells. For each coverslip, greater than 300 cells were counted.

Quantitative RT-PCR

RNA was isolated from infected macrophages using the RNeasy Mini kit and DNase-treated using the RNase-free Dnase set (Qiagen) to remove contaminating genomic DNA. The isolated RNA was then reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen). Relative mRNA abundance was measured by qPCR using SYBR Green and the CFX96 qPCR machine (Bio-Rad Laboratories). The following primer pairs were used: *Nos2* 5' (ACATCGACCCGTCAACAGTAT), *Nos2* 3' (CAGAGGGGTAGGCTTGTCTC), *mGBP1* 5' (AGATGCCCTTGGTGTGAGAC), *mGBP1* 3' (CTCTACCGCACAGGCAATC), *mGBP2* 5' (TCTACCCCACTCTGGTCAGG), *mGBP2* 3' (CAGCATAGGAACCATCAACCA), *Hprt* 5' (GTTGGATACAGGCCAGACT), *Hprt* 3' (GAGGGTAGGCTGGCCTAT). To calculate relative fold-induction using the $\Delta\Delta CT$

method (Shin et al., 2008), the cycle threshold (Ct) of a given gene was normalized to HPRT Ct and compared to the normalized Ct in uninfected cells.

Measurement of Nitric Oxide

Macrophages were infected at indicated MOIs in 96 well plates at 1.5×10^5 cells per well in 150 μ L media. At indicated time points, nitrite (NO_2^-) levels were measured with the Griess reagent system according to the manufacturers guidelines (Promega).

Measurement of Whole Cell ROS

6.0×10^5 macrophages were plated in 12-well non-tissue culture plates. Cells were treated with 0.5 μ g/mL for 6 hours or infected with WT *C. burnetii* MOI=100 for 16 hours. Cells were incubated with 2.5 μ M CM-H2DCFDA and HBSS containing Ca, Mg, and glucose for 20 min in 37° C for 20 minutes in a humidified incubator. Levels of whole cell ROS were measured by flow cytometry.

Statistical Analysis

The plotting of data and statistical analysis were performed using GraphPad Prism software. Statistical significance was determined using unpaired, two-tailed Student's *t* test or one-way analysis of variance (ANOVA) with Tukey's posttest. Differences were considered significant if the *P* value was <0.05.

Results

TNF is critical for cell-intrinsic control of *Coxiella burnetii* Nine Mile phase II replication.

The addition of exogenous TNF along with IFN γ to a mouse fibroblast cell line or human monocytes has been shown to restrict intracellular *C. burnetii* replication (Ghigo et al., 2001; Howe et al., 2002). Furthermore, a previous study showed that antibody-mediated neutralization of TNF led to increased *C. burnetii* replication in human monocytes (Howe et al., 2002). Whether endogenous levels of TNF produced by B6 BMDMs during infection contribute to the restriction of intracellular *C. burnetii* replication is unknown; moreover, it is unknown if TNF alone is sufficient to inhibit intracellular replication in this cell type. To test this, we infected *Tnf*^{-/-} BMDMs with WT *C. burnetii* and measured bacterial uptake (Figure 3.6A), intracellular replication (Figure 3.1A), and vacuole formation (Figures 3.1C and 3.1D). We observed robust intracellular bacterial growth in *Tnf*^{-/-} BMDMs, with a greater than 25-fold increase in GE and an increase in *C. burnetii*-containing vacuoles by day seven post-infection, suggesting that endogenously produced TNF plays a major role in the ability of B6 BMDMs to restrict *C. burnetii* replication. To confirm this restriction was mediated by soluble TNF, we added 10ng/mL recombinant TNF to *C. burnetii*-infected *Tnf*^{-/-} BMDMs at days 1 and 5 post-infection (Figures 3.1B-D). We found that the addition of recombinant TNF restored the ability of *Tnf*^{-/-} BMDMs to restrict *C. burnetii* replication, as measured by a decrease in GEs and the percentage of *C. burnetii* vacuoles, suggesting that the deficiency in TNF production accounts for the increased susceptibility of *Tnf*^{-/-} BMDMs, and that soluble TNF is sufficient to inhibit *C. burnetii* replication in these cells. Furthermore, the addition of supernatants from B6 BMDMs infected with *C. burnetii* for 24 hours restored the ability of *Tnf*^{-/-} BMDMs to restrict intracellular *C. burnetii* growth, further supporting that intracellular restriction of *C. burnetii* replication by B6 BMDMs is mediated by secreted factor(s), one of which is TNF. Finally, we examined whether the defect in the ability of

Tlr2^{-/-} BMDMs to control *C. burnetii* could be complemented by exogenous treatment with rTNF or the supernatants from *C. burnetii*-infected B6 BMDMs (Figure 3.1E). In contrast to mock-treated *Tlr2*^{-/-} BMDMs, *Tlr2*^{-/-} macrophages treated with rTNF or supernatants from *C. burnetii*-infected BMDMs were able to restrict intracellular *C. burnetii* replication to levels comparable to B6 BMDMs. Taken together, our data suggest that endogenous TNF produced in response to TLR sensing of *C. burnetii* infection critically contributes to the ability of B6 macrophages to control *C. burnetii* replication. Furthermore, these data indicate that the inability of *Tlr2*^{-/-} BMDMs to control *C. burnetii* replication is primarily due to a defect in TNF production, as addition of exogenous TNF restored the ability of these cells to limit intracellular *C. burnetii* replication.

***Coxiella burnetii* phase II does not induce cell death in B6 macrophages**

Cell death is one mechanism by which cells control pathogens. TNF can induce both non-inflammatory apoptosis and inflammatory necrosis. One study in THP-1 cells found that *C. burnetii* induces cell death 24 hours post infection (Zhang et al., 2012), while another study showed that *C. burnetii* inhibits extrinsic apoptosis, which is mediated by TNF signaling to caspase-8 (Voth et al., 2007). Cell death in B6 BMDMs infected with *C. burnetii*, however, has not been studied. We found no cell death in *C. burnetii*-infected B6 macrophages using the LDH bulk assay at 24 hours post-infection (Figure 2.6C), but wanted to test with a more sensitive single cell assay whether cell death was occurring. In order to determine if TNF-dependent cell death occurs and is a possible mechanism of control for *C. burnetii*, we employed the TUNEL method of staining. We were unable to detect cell death at 24 hours post-infection at an MOI=100, the same MOI used in our growth curves (Figure 3.2A and 3.2B). These data, coupled with our previous findings

using the LDH cytotoxicity assay (Figure 2.6C), suggest that TNF-mediated cell death is not a major mechanism by which B6 macrophages control *C. burnetii*.

***Coxiella burnetii* infection induces the production of iNOS and Reactive Nitrogen Species (RNS) in B6 macrophages in a TNF-dependent manner**

Reactive nitrogen species (RNS) are an additional TNF-mediated cell-intrinsic immune response. TNF in conjunction with IFN γ have been shown to induce RNS, and this induction has been shown to play a role in the clearance of *C. burnetii* (Howe et al., 2010). TNF alone, however, has never been implicated in RNS-mediated restriction of *C. burnetii*. Here, we measured induction of iNOS, the protein that synthesizes RNS through its substrate L-arginine. We found *C. burnetii* does induce expression of *Inos* mRNA via qRT-PCR and this expression is partially dependent on endogenously-produced TNF (Figure 3.3A). We further tested if *C. burnetii* induced formation of a functional NOS2 protein by measuring levels of RNS in the supernatant. *C. burnetii* infection did induce RNS and that induction was partially dependent on endogenous TNF (Figure 3.3B). Interestingly, exogenous TNF alone was sufficient to induce high levels of *Inos* transcript, but was not sufficient to induce dimerization and form a functional iNOS protein to generate measurable levels of NO $_2^-$, indicating that additional stimuli are necessary for assembly of functional iNOS proteins. These data demonstrate that RNS is generated by *C. burnetii* in a TNF-dependent manner, offering one potential mechanism by which TNF restricts *C. burnetii* in B6 macrophages.

***Coxiella burnetii* phase II infection induces ROS, which is a potential mechanism of bacterial restriction in B6 macrophages**

ROS is another cell-intrinsic control mechanism that can be induced by TNF. Some studies show *C. burnetii* does not induce an oxidative burst, while other studies have demonstrated that ROS is produced in response to *C. burnetii* infection (Akporiaye et al., 1990; Brennan et al., 2004; Howe et al., 2002). To determine if *C. burnetii* can induce ROS in C57BL/6 macrophages, we infected BMDMs and measured whole cell ROS at 16 hours post-infection by flow cytometry using the reagent H2DCFDA, which is nonfluorescent but is converted to a green-fluorescent form when oxidation by the activity of ROS occurs within the cell. We found that *C. burnetii* does induce measurable ROS production (Figure 3.4A). To test if ROS was able to inhibit *C. burnetii* growth, we measured growth in *gp91phox*^{-/-} macrophages and found significantly increased growth in the absence of this component of NADPH oxidase (Figure 3.4B). These data provide preliminary evidence for an additional TNF-mediated mechanism by which B6 BMDMs restrict *C. burnetii* replication.

***Coxiella burnetii* phase II infection induces expression of Guanylate Binding Proteins, but they do not play major role in control of bacterial replication**

Guanylate binding proteins (GBPs) are a class of interferon-inducible GTPases with a variety of functions and have been implicated in the control of intracellular pathogens. It is known that in conjunction with IFN γ , TNF can induce robust expression of all 11 murine GBPs (Olszewski et al., 2006; Vestal and Jeyaratnam, 2011; Yamamoto et al., 2012); however, the role of TNF alone as an inducer of GBP expression remains poorly understood. Moreover, a possible role for GBPs in the context of *C. burnetii* infection has not been assessed. Here, we tested the induction of two murine GBPs, mGBP-1 and mGBP-2, which have been shown to exhibit antimicrobial properties via delivery of anti-

microbial products to autolysosomes. We found that the mRNAs encoding mGBP-1 and mGBP-2 are induced in B6 BMDMs by TNF alone (Figure 3.5A-B). Furthermore, we found that *C. burnetii* infection induces transcription of these GBPs in a dose-dependent manner (Figures 3.5A and 3.5B). To test if these or other GBPs played a role in *C. burnetii* clearance, we measured bacterial replication in *GBP^{Chrom3-/-}* BMDMs (Yamamoto et al., 2012), which are deficient in mouse GBPs 1, 2, 3, 5, and 7. We did not observe any difference in bacterial replication in GBP-deficient macrophages (Figure 3.5C), ruling out these five GBPs as mediators of *C. burnetii* restriction.

Discussion

Previously we found a major role for the cell surface immune sensors TLR2 and TLR4 as mediators to restrict *C. burnetii* replication in B6 BMDMs. We also observed induction of pro-inflammatory cytokines to be dependent on TLR2 and TLR4, and rely on both Myd88 and Trif adaptor proteins. Since TNF has a known role as a mediator of cell-intrinsic immunity against other bacterial pathogens, we hypothesized that TNF induces multiple cell-intrinsic pathways to restrict *C. burnetii* replication. In this study, we found that TNF has a major role in the restriction of *C. burnetii*. We further investigated three potential mechanisms of TNF-mediated control: (1) cell death; (2) reactive nitrogen (RNS) and reactive oxygen (ROS) species; and (3) guanylate-binding proteins (GBPs), and preliminarily found a role for ROS in controlling *C. burnetii* replication.

TNF is a pleotropic cytokine that induces a wide array of cell-extrinsic and cell-intrinsic proinflammatory responses (Grell, 1995; Wajant et al., 2003). Since TNF is a major player in inflammation and underlies the pathogenesis of many autoimmune diseases, researchers have developed a host of anti-TNF therapies (Taylor, 2001). The

widespread use of these therapies has revealed their use leads to higher susceptibility to a variety of interacellular pathogens, including *M. tuberculosis* and *L. pneumophila* (Jinno et al., 2009; Wagner et al., 2002). In our study we sought to elucidate the cell-intrinsic mechanism by which TNF inhibits *C. burnetii* replication in an effort to better understand how this broad-acting cytokine restricts intracellular bacterial infection.

We first wanted to determine if *C. burnetii* infection induces these pathways. If induced, we then wanted to determine if this induction was dependent on TNF and if the pathway played a role in restricting of bacterial replication. We found that *C. burnetii* does induce RNS, ROS, mGBP-1, and mGBP-2 in B6 macrophages, but does not induce cell death. We then investigated if TNF induces measurable cell death in B6 macrophages. TNF can induce both non-inflammatory extrinsic apoptosis and inflammatory necrosis (Schulze-Osthoff et al., 1998; Vandenabeele et al., 2010). Both of these cell death pathways are thought to control intracellular pathogens by eliminating their replicative niche. Programmed necrosis also has the potential to alert neighboring immune cells by releasing DAMPs, which stimulate TLRs and cytosolic sensors (Pasparakis and Vandenabeele, 2015). In the context of *C. burnetii* infection, whether cell death is induced or whether it restricts bacterial infection is unclear. A previous study demonstrated that *C. burnetii* inhibits extrinsic apoptosis. In this study, THP-1 cells were infected for 48 hours and then treated with TNF and cyclohexamide to artificially induce caspase-8-mediated apoptosis (Voth et al., 2007). They found reduced PARP cleavage in infected cells compared to uninfected cells. Another study in THP-1 cells found that cell death does occur in the first 24 hours of *C. burnetii* infection. This cell death was caspase-3-independent and TNF-dependent (Zhang et al., 2012). This latter study suggests that TNF mediated-cell death may be a mechanism of control, whereas

the earlier study suggests *C. burnetii* has evolved strategies to evade these control mechanisms. Here, we determined whether B6 macrophages undergo cell death during *C. burnetii* infection. We measured cell death via both the LDH release assay, which broadly measures general cell death at the population level, and the TUNEL assay, which is primarily used to detect apoptotic cell death at the single cell level. In both assays we found no measurable cell death at 24 hours post infection. Since TUNEL does not stain well for cells undergoing programmed necrosis (Nonaka et al., 2003), these data do not completely rule out necroptosis as an early mechanism of restriction. Further studies using propidium iodide staining, which stains both apoptotic and necroptotic cells or growth curves in *Rip3*^{-/-} macrophages are necessary to rule out programmed necrosis as a mechanism of *C. burnetii* control (Nonaka et al., 2003; Vandenabeele et al., 2010). Given the LDH bulk assay showed no measurable death, it is unlikely that necroptosis is robustly induced, if at all.

Our data rule out TNF-mediated apoptosis as a major mechanism of bacterial control since we observed that death is not robustly induced during *C. burnetii* infection. A previous study indicated that artificially induced TNF-mediated extrinsic apoptosis is inhibited by *C. burnetii* in human THP-1 cells (Voth et al., 2007), but no direct link to bacterial effectors was made. Complete inhibition by the T4SS alone is unlikely given that we see no measurable cell death and we would expect that bacterial effector-driven inhibition of cell death would not be one hundred percent complete. A more likely reason is that, unlike *Yersinia spp.* or *Mycobacterium avium*, *C. burnetii* does not induce extrinsic apoptosis or alternatively, *C. burnetii* induces an anti-apoptotic signaling pathway (Fratazzi et al., 1997; Ruckdeschel et al., 1997). Future studies with the T4SS-deficient strain and the exogenous induction of TNF-mediated apoptosis would

determine if *C. burnetii* is actively inhibiting this pathway. To conclusively determine whether this pathway plays a role in the restriction of *C. burnetii* replication in B6 macrophages, *C. burnetii* replication could be assessed in *Rip3^{-/-}Casp8^{-/-}* BMDMs, which are unable to undergo either extrinsic apoptosis or necroptosis (Pasparakis and Vandenabeele, 2015).

We also examined ROS and RNS as possible effector mechanisms of TNF-mediated control of *C. burnetii* infection in B6 macrophages. ROS and RNS have previously been shown to be induced by *C. burnetii* infection in some studies and not induced in others (Akporiaye et al., 1990; Brennan et al., 2004; Dellacasagrande et al., 2000; Zamboni and Rabinovitch, 2003). In the presence of exogenous TNF and IFN γ , ROS and RNS were implicated in the restriction of *C. burnetii* replication (Zamboni and Rabinovitch, 2003). However, it has also been shown that *C. burnetii* secretes an acid phosphatase, which may play a role in resistance to ROS (Hill and Samuel, 2011). Whether endogenously produced TNF induces production of ROS and RNS in C57BL/6 macrophages during *C. burnetii* infection has not been shown. Moreover, whether RNS and ROS mediate restriction of *C. burnetii* in macrophages in the absence of exogenously added recombinant cytokines has not been extensively studied.

We found *Nos2* mRNA and the iNOS byproduct NO $_2^-$ were induced in B6 macrophages in response to *C. burnetii* infection, and we observed a significant reduction in the induction of both *Nos2* and RNS in *Tnf^{-/-}* macrophages. These findings indicate that *C. burnetii* does induce a functional iNOS protein that is partially dependent on the production of endogenous TNF. *Tnf^{-/-}* macrophages still produced a low level of iNOS and NO $_2^-$, which suggests that another pathway, potentially TLR signaling itself, can lead to the production of RNS during *C. burnetii* infection. To determine if iNOS

plays a role in restriction of *C. burnetii*, measuring bacterial growth in *iNOS*^{-/-} BMDMs or growth in the presence of iNOS inhibitors, such as S-methylisothiourea sulfate and L-N(6)-(1-iminoethyl)-lysine (L-NIL) are necessary.

We also demonstrated *C. burnetii* infection results in production of measurable levels of whole cell ROS by B6 macrophages. Furthermore, our preliminary results indicate that gp91phox, a component of NADPH oxidase, plays a role in restricting *C. burnetii* growth in B6 macrophages. These data indicate that ROS is induced during infection and mediate a role in growth restriction, but do not show that this ROS is the direct product of TNF signaling. Since ROS can be induced by a variety of stimuli and pathways, future studies should analyze ROS production in *Tnf*^{-/-} BMDMs to determine if induction is dependent on this cytokine or via another mechanism.

Interferon-inducible guanylate-binding proteins (GBPs) have recently been implicated in the cell-intrinsic control of a variety of pathogens and have been shown to be induced by TNF (Pilla et al., 2014; Vestal and Jeyaratnam, 2011; Yamamoto et al., 2012). Their role in host control of *C. burnetii* infection has not been assessed. Moreover, it is not known whether TNF alone, in the absence of interferon, is sufficient to induce expression of many mGBPs. Here we demonstrated that either TNF alone or *C. burnetii* infection are sufficient to induce expression of the mRNAs encoding mGBP-1 and mGBP-2, which have been shown to exhibit antiviral and antiparasitic responses by delivery of antimicrobial cargo to the autophagosome (Degrandi et al., 2013; Vestal and Jeyaratnam, 2011). We further tested if mGBP-1,-2,-3,-5, and -7 play a role in growth restriction using mice missing these GBPs, which are encoded on chromosome 3 (Yamamoto et al., 2012). We found that these five murine GBPs were not required to restrict *C. burnetii* replication. Induction of mouse GBPs present on chromosome 5,

mGBP-6,-8,-9,-10,-11 (mGBP-4 is non-functioning in C57BL/6 macrophages) and whether these GBPs are localized to the *C. burnetii* vacuole should also be tested. If *C. burnetii* does induce these GBPs, then experiments examining the roles of these GBPs during *C. burnetii* infection using siRNA-mediated gene silencing could shed light on their role in restricting bacterial replication.

In conclusion, we were able to build upon our previous findings that TLR detection of *C. burnetii* infection is a major mechanism of control in C57BL/6 macrophages. Furthermore, we were able to implicate TNF produced as a consequence of TLR detection as a major player in this restriction. In our analysis of possible TNF-mediated restriction mechanisms, our preliminary data indicate that the NADPH oxidase plays a role in the inhibition of *C. burnetii* replication in C57BL/6 macrophages.

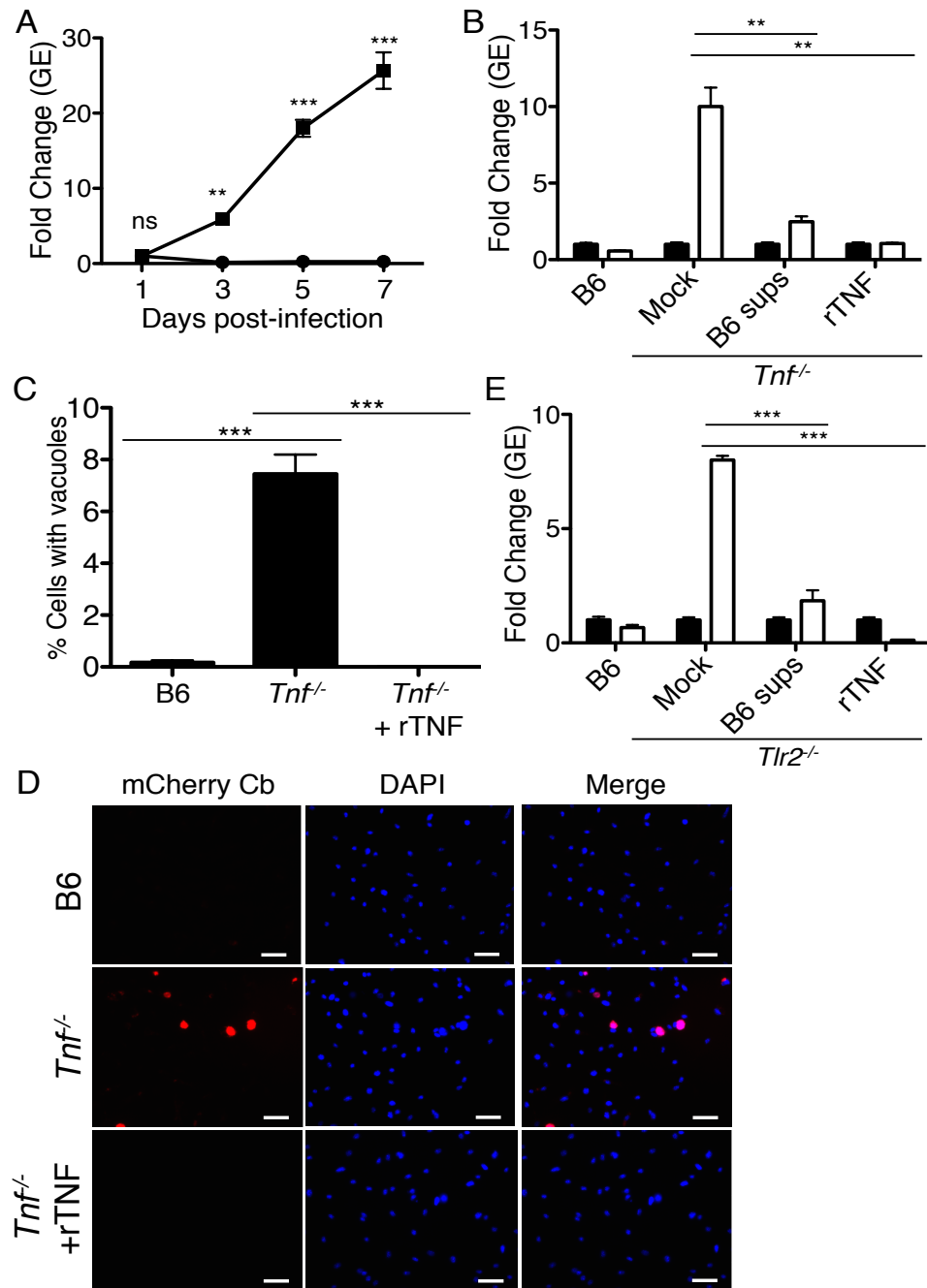


Figure 3.1 Endogenous TNF restricts intracellular growth of *C. burnetii*. (A)

C57BL/6 and *Tnf*^{-/-} BMDMs were infected with WT *C. burnetii* NMII expressing mCherry at MOI=100. *C. burnetii* GEs were determined by qPCR on days 1, 3, 5, and 7 post-infection. Graphs show the fold change in GEs relative to day 1 ± SEM of triplicate wells.

Representative of three independent experiments. (B) 10ng/mL rTNF or supernatants from C57BL/6 BMDMs infected with WT *C. burnetii* NMII for 24 hours were added to WT *C. burnetii*-infected *Tnf*^{-/-} and *Tlr2*^{-/-} BMDMs on days 1 and 5 post-infection. *C. burnetii* GEs were measured by qPCR and the fold increase in GEs on day 7 post-infection was determined relative to the GEs on Day 1. Graphs show the fold change in GEs relative to day 1 ± SEM of triplicate wells. Representative of two independent experiments. (C) B6 or *Tnf*^{-/-} BMDMs were infected with mCherry-expressing WT *C. burnetii* or 10ng/mL rTNF was added to mCherry-expressing WT *C. burnetii*-infected *Tnf*^{-/-} BMDMs on days 1 and 5 post-infection. On day 7 post-infection, cells were fixed, stained with DAPI, and imaged by fluorescence microscopy. The number of mCherry-expressing *C. burnetii*-containing vacuoles was determined and calculated as a percentage of total cell number. Graphs show mean percentage of cells containing *C. burnetii* vacuoles ± SEM of triplicate coverslips. At least 300 cells were counted per coverslip. (D) Representative images (40x) of mCherry-expressing *C. burnetii*-containing vacuoles in infected BMDMs treated as in (C). Scale bars represent 25μM. Representative of two independent experiments. (E) 10ng/mL rTNF or supernatants from B6 BMDMs infected with WT *C. burnetii* NMII for 24 hours were added to WT *C. burnetii*-infected *Tlr2*^{-/-} BMDMs on days 1 and 5 post-infection. *C. burnetii* GEs were measured by qPCR and the fold increase in GEs on day 7 post-infection was determined relative to the GEs on Day 1. Graphs show the fold change in GEs relative to day 1 ± SEM of triplicate wells. Representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001, ns=no significance.

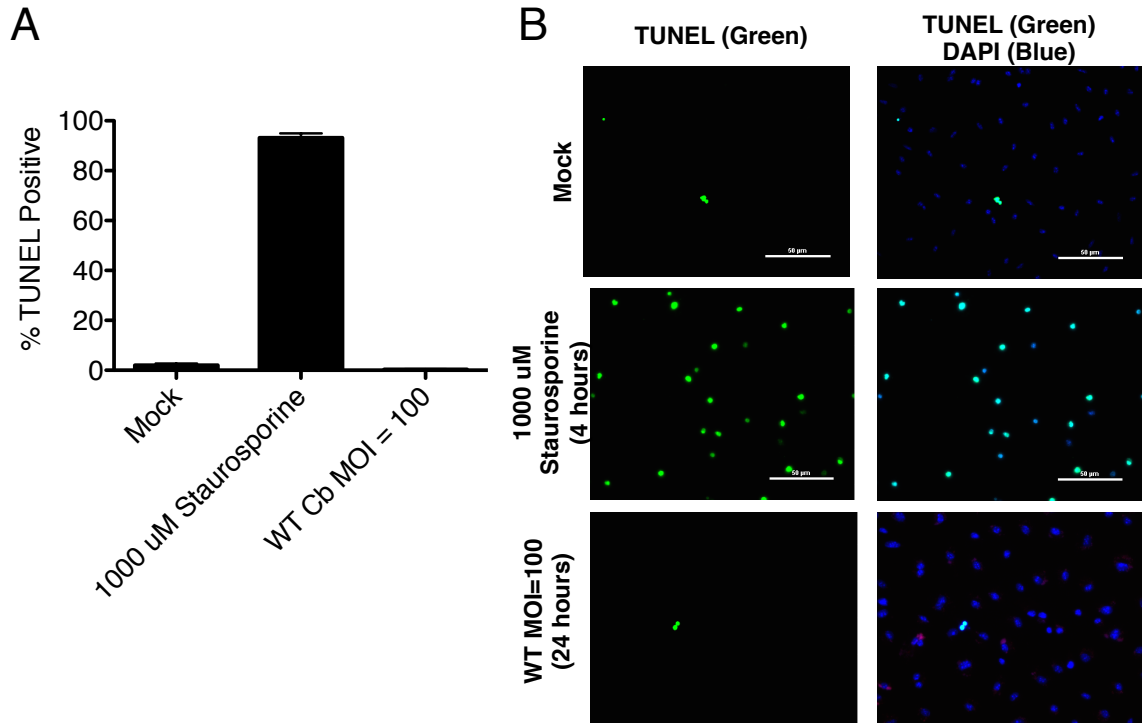


Figure 3.2 *C. burnetii* does not induce apoptosis in B6 macrophages. (A) and (B)

C57BL/6 BMDMs were infected with WT *C. burnetii* NMII expressing mCherry at MOI=100 for 24 hours or treated with 1000 μ M staurosporine for 4 hours. Post-infection, cells were fixed, stained with TUNEL (green) and DAPI (blue), and imaged by fluorescence microscopy. The number of TUNEL positive cells was determined and calculated as a percentage of total cell number. Graphs show mean percentage of TUNEL positive cells \pm SEM of triplicate coverslips. At least 300 cells were counted per coverslip. (B) Representative images (20x) of TUNEL positive cells positive cells.

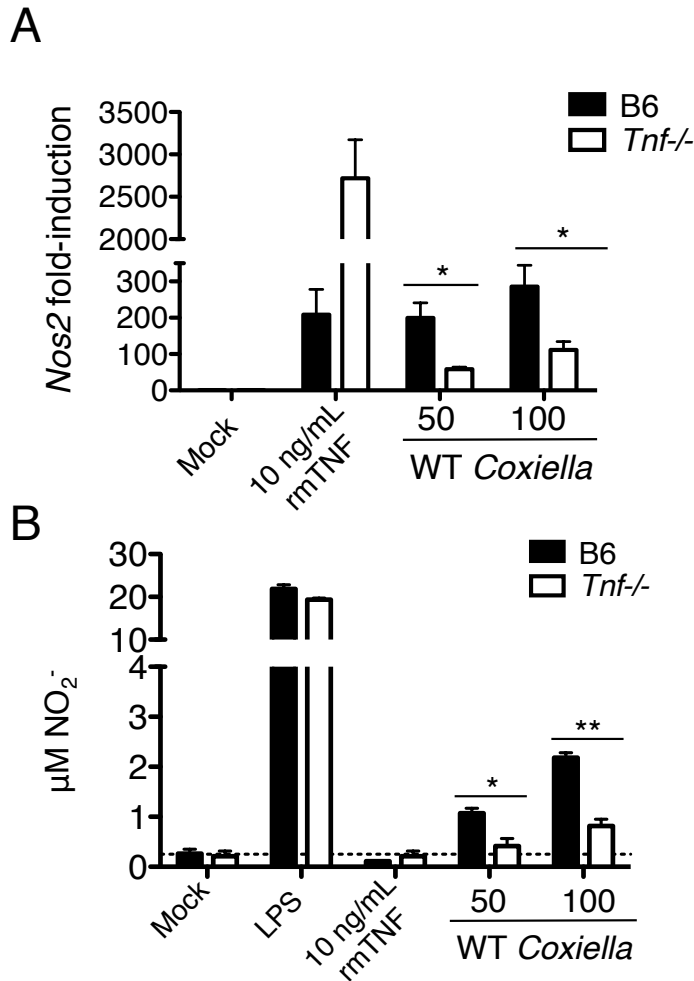


Figure 3.3 *C. burnetii* infection does induce iNOS mRNA and NO_2^- in a TNF

dependent manner. C57BL/6 and *Tnf*^{-/-} BMDMs were mock-infected, treated with *E. coli*

LPS, 10 ng/mL mouse recombinant TNF, or infected with WT *C. burnetii* NMII at MOI=

50 or 100. (A) 16 hours post infection, fold-induction of *Nos2* mRNAs were measured via qRT-PCR. Graphs show the mean \pm SEM of triplicate wells in a 48 well plate.

Representative of two independent experiments. (B) NO_2^- was measured in the

supernatants 24 hours post infection. Graphs show the mean \pm SEM of triplicate wells in

a 96 well plate. Representative of two independent experiments. *p<0.05, **p<0.01.

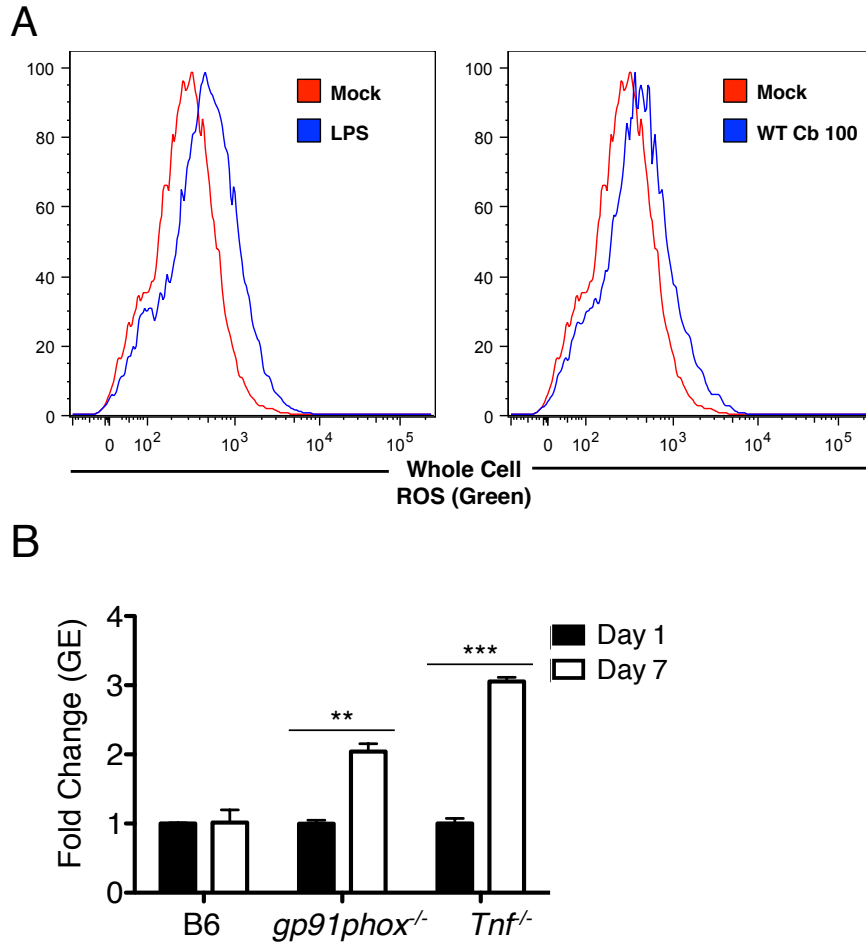


Figure 3.4 *C. burnetii* infection induces whole cell ROS and ROS plays a role in restricting intracellular growth in B6 BMDMs. (A) C57BL/6 were mock-infected, treated with *E. coli* LPS for 6 hours, or infected with WT *C. burnetii* NMII at MOI= 100 for 16 hours. Whole cell ROS was measured by flow cytometry. Representative of two independent experiments. *p<0.05, **p<0.01. (B) C57BL/6, *gp91phox*^{-/-}, and *Tnf*^{-/-} BMDMs were infected with WT *C. burnetii* NMII expressing mCherry at MOI=100. *C. burnetii* GEs were determined by qPCR on days 1, 3, 5, and 7 post-infection. Graphs show the fold change in GEs relative to day 1 ± SEM of triplicate wells. **p<0.01 and ***p<0.001.

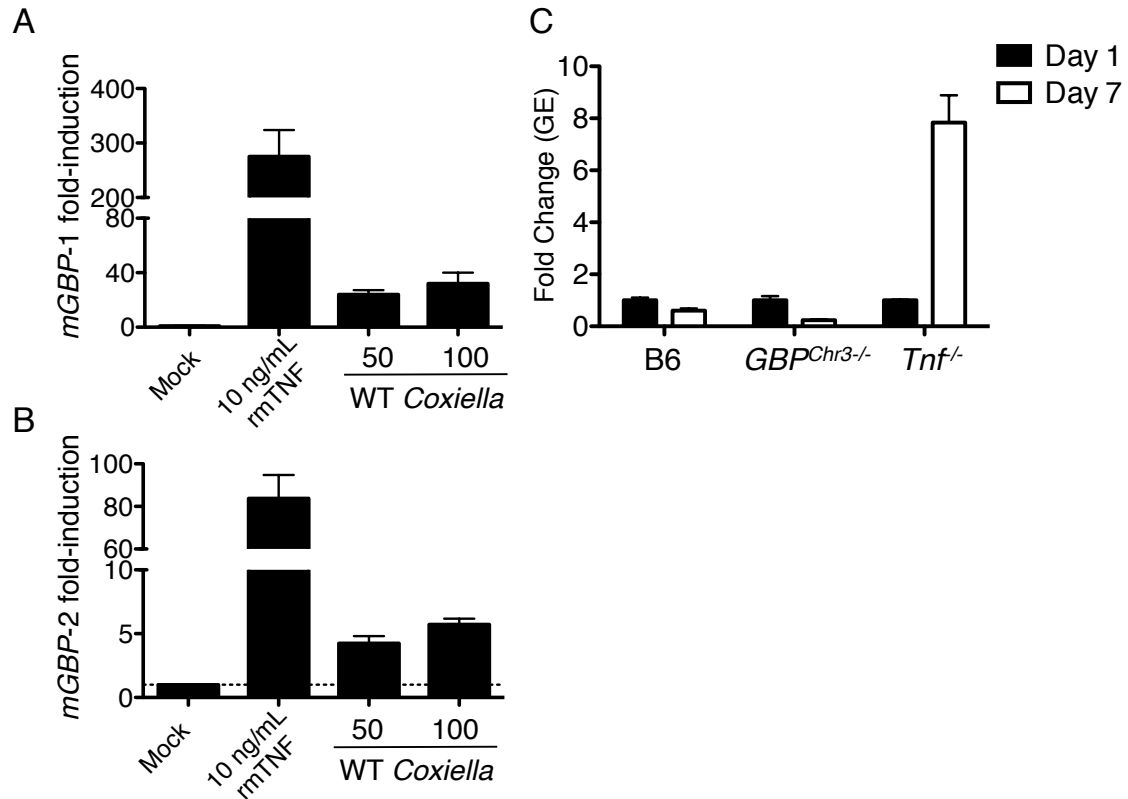


Figure 3.5 *C. burnetii* infection induces expression of mGBP-1 and mGBP-2, but GBPs 1, 2, 3, 5, and 7 do not play a role in restricting *C. burnetii* replication. (A) and (B) C57BL/6 BMDMs were mock-infected, 10 ng/mL recombinant mouse TNF, or infected with WT *C. burnetii* NMII at MOI= 50 or 100. 16 hours post infection, fold-induction of *mGBP-1* and *mGBP-2* mRNAs were measured via qRT-PCR. Graphs show the mean \pm SEM of triplicate wells in a 48 well plate. Representative of two independent experiments. (C) C57BL/6, *GBP^{Chr3-/-}* and *Tnf^{-/-}* were infected with WT *C. burnetii* NMII expressing mCherry at MOI=100. *C. burnetii* GEs were determined by qPCR on days 1, 3, 5, and 7 post-infection. Graphs show the fold change in GE relative to day 1 \pm SEM of triplicate wells. Graphs show the mean \pm SEM of triplicate wells.

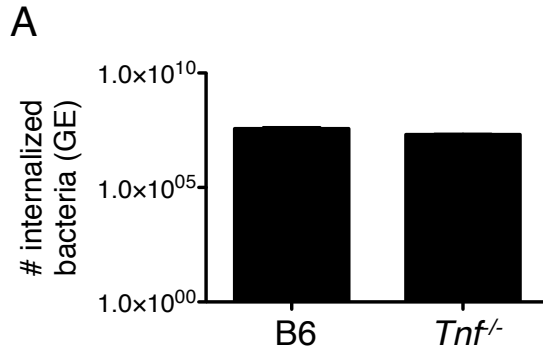


Figure 3.6. Levels of internalized WT *Coxiella burnetii* are similar for different genotypes of bone marrow-derived macrophages at 24 hours post-infection. 24 hours post-infection, the number of internalized WT *C. burnetii* NMII genomic equivalents (GEs) were measured by qPCR for (A) C57BL/6, *Tlr2*^{-/-}, *Tlr4*^{-/-}, and *Tlr2*^{-/-}*Tlr4*^{-/-} BMDMs presented in Figure 2.1C,

CHAPTER 4:

DISCUSSION

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In the studies described here, we sought to determine the innate immune response mounted by macrophages against *C. burnetii burnetii*, a poorly understood intracellular bacterium. The *C. burnetii* field has made great advances over the years. The advent of an axenic growth media and improved methods for genetic manipulation has given the scientific community new tools to better understand its pathogenesis. While the field has made considerable progress in understanding the cell biology of *C. burnetii*'s intracellular niche, the innate immune response to *C. burnetii* is poorly understood. This is in part due to wide variation in cell lines, animal models, and bacterial strains employed by different groups.

Our first goal was to establish a standardized system in C57BL/6 primary bone marrow-derived macrophages. This system has been used to study a variety of pathogens and due to the large number of available mice with select innate immune deficiencies, allowed us to cast a wide net in the innate immune pathways we analyzed. Furthermore, in the context of the *C. burnetii* Nine Mile phase II strain, this system offers a unique opportunity to study cell-intrinsic mechanism of bacterial control as C57BL/6 macrophages are naturally able to restrict *C. burnetii* NMII infection.

With this infection system, in our first aim we sought to answer two questions: [1] which innate immune responses are activated *in vitro*; and [2] what role does Type IV secretion system-mediated translocation of bacterial products play in triggering these innate immune responses. We hypothesized that since *C. burnetii* is able to survive inside cells over a long period of time and delays neutrophil recruitment in the lung (Elliott et al., 2013; van Schaik et al., 2013), it evolved strategies to evade innate immune detection, potentially through the injection of T4SS effector proteins. Our survey of three classical innate immune pathways allowed us to implicate TLR-mediated TNF

production as a major player in the cell-intrinsic control of *C. burnetii* in C57BL/6 macrophages.

TLR detection is more critical than cytosolic detection for the induction of pro-inflammatory cytokines and the cell-intrinsic control of *Coxiella*

To address our first aim, we assessed *C. burnetii*'s ability to activate various innate immune pathways in C57BL/6 BMDMs. To further test our hypothesis that *C. burnetii* has evolved strategies to subvert innate immune detection we investigated the role of its T4SS in activating these pathways. In particular, we examined cell surface and cytosolic immune detection and induction of pro-inflammatory cytokines, the activation of the inflammasome, and the induction of Type I interferon response. We wanted to both determine *C. burnetii*'s ability to activate these pathways and test whether they mediated cell-intrinsic control of bacterial replication (Zamboni, 2004). Furthermore, to determine the role of the T4SS in mediating these responses, we utilized an isogenic NMII strain containing a transposon insertion in the gene *icmL* (Carey et al., 2011).

In these studies, we built upon past work that showed an important role for TLR2 in mediating production of pro-inflammatory cytokines and an inconclusive role for TLR4 (Barry et al., 2012; Shannon et al., 2005; Zamboni et al., 2004). We confirmed previous findings that TLR2 plays a major role in the production of pro-inflammatory cytokines TNF and IL-6, but also found a role for TLR4 in the induction of these cytokines. Upon further analysis of the downstream adaptors MyD88 and Trif, we found a role for both MyD88 and Trif in both the production of TNF and IL-6 and the inhibition of *C. burnetii* replication. Since Trif is classically thought to signal only through TLR4 and TLR3 (Akira, 2003), with TLR3 largely associated with recognition of viral infection, this further

suggests TLR4 responds to *C. burnetii* infection. We wanted to next determine whether *C. burnetii*'s LPS was the ligand responsible for TLR4-dependent responses. Past studies have shown that purified *C. burnetii* LPS is not stimulatory for TLR4 and instead acts as a TLR4 antagonist (Zamboni et al., 2004). We confirmed this was the case in our system. TLR4 can also recognize host danger-associated molecular patterns (DAMPs) that are released upon infection such as HMGB-1 (Pasparakis and Vandenabeele, 2015), but these are unlikely since we observe no measurable cell death 24 hours post infection. One possibility is that TLR4 recognition of *C. burnetii* LPS requires simultaneous detection of other bacterial PAMPs by other TLRs.

Since the T4SS is required for *C. burnetii* growth within host cells (Beare et al., 2011), it is possible that restrictive C57BL/6 macrophages are somehow not permissive for translocation of T4SS substrates, and thus cytosolic immune sensors would not be triggered during *C. burnetii* infection in this cell type. We confirmed via a single cell assay that the *C. burnetii* T4SS does inject effectors into C57BL/6 macrophages, allowing us to further investigate whether cytosolic immune detection occurs. To examine this, we first compared the ability of WT NMII and *icmL::Tn* NMII, lacking a functional T4SS, to induce proinflammatory cytokines. The *L. pneumophila* T4SS has been shown to trigger an enhanced cytokine response, presumably due to its translocation of multiple bacterial ligands that are detected by cytosolic sensors that promote cytokine expression. We interestingly found no increase in TNF or IL-6 production by B6 macrophages infected with WT bacteria compared to the *icmL::Tn* mutant, and our data suggest that there is a slight decrease in TNF and IL-6 production in response to WT bacteria. This suggests that *C. burnetii* either does not secrete immunostimulatory ligands or that it inhibits cytokine production via one or more T4SS

effectors. Future studies will determine if *C. burnetii* T4SS effectors are actively blocking TNF and IL-6 production. Since *C. burnetii* has undergone genome reduction over its evolutionary history, it is also possible that it has altered or eliminated genes encoding immunostimulatory ligands.

To further test whether there is cytosolic immune detection of *C. burnetii*, we investigated *C. burnetii*'s ability to activate the inflammasome. To test this, we used a variety of readouts for inflammasome activation that have been employed to study the inflammasome in the context of other bacterial pathogens, such as *L. pneumophila* (Casson and Shin, 2013). We surprisingly found no activation of the inflammasome following *C. burnetii* infection of B6 macrophages, even in cells that had been primed with LPS to ensure that all the necessary inflammasome components were highly upregulated. This phenomenon could again be the result of *C. burnetii* not encoding inflammasome-activating ligands or actively inhibiting inflammasome activation via T4SS effectors, as seen with *Yersinia* species, which utilize the T3SS effectors YopK and YopM to inhibit caspase-1 activation (Brodsky et al., 2010; LaRock and Cookson, 2012). Future studies that examine whether *C. burnetii* can inhibit inflammasome activation downstream of external stimuli, similar to those used to determine that *C. burnetii* inhibits intrinsic apoptosis, would offer further insight into *C. burnetii*'s subversion of the inflammasome.

Type I interferon (IFN) was the final arm of the innate immune response we studied. Type I IFNs are thought to primarily play a role in the immune response to viruses and have the potential to be induced by immune sensors both at the cell surface and within the cytosol (Müller et al., 1994). Recently, they have been shown to play a role in some bacterial infections (Lippmann et al., 2011; Monroe et al., 2010). Studies have shown

type I IFNs can restrict *Legionella* replication (Coers et al., 2007; Schiavoni et al., 2004), while other studies have shown that they can promote bacterial infection, as is the case for *M. tuberculosis* (Manzanillo et al., 2012). Whether type I IFNs play a role in restricting *C. burnetii* infection has not been studied. Despite a role for Trif, which induces type I IFN production downstream of IRF-3 phosphorylation (Akira, 2003), in both cytokine production and bacterial restriction, we did not observe robust induction of Type I interferon, nor did we observe a role for type I IFNs in the restriction of *C. burnetii* replication in B6 macrophages.

In sum, we determined that TLR detection plays a dominant role in the production of proinflammatory cytokines during *C. burnetii* infection. Although we found that the T4SS is functional and translocates bacterial products into B6 macrophages, our data rule out cytosolic immune activation of pro-inflammatory cytokines and the inflammasome as major players mediating the restriction of *C. burnetii* growth in B6 macrophages. We further ruled out the type I IFN response as being induced or playing a role in cell-intrinsic restriction during *C. burnetii* infection. It again is possible that *C. burnetii* inhibits type I IFN induction through the activity of its T4SS effectors or does not possess or expose immunostimulatory ligands that robustly activate the sensors leading to type I IFN induction. However, since Trif plays an important role in TNF and IL-6 induction during *C. burnetii* infection, we would have anticipated that Trif signaling would have also led to type I IFN induction. In future studies, it would be of interest to determine if IRF3 is phosphorylated. If IRF3 is activated, then it is possible that *C. burnetii* may target the transcription or translation of type I IFNs.

TNF is a mechanism by which B6 macrophages inhibit *Coxiella burnetii* replication.

We expanded upon our initial studies, which implicated TLRs as the major sensors responsible for production of cytokines during *C. burnetii* infection and cell-intrinsic control of bacterial replication. We hypothesized that TLR signaling induces a soluble factor that then activates an antimicrobial response. We found TNF, a pleiotropic cytokine that activates many cell-intrinsic and cell-extrinsic immune pathways, plays a major role in *C. burnetii* restriction. TNF has been implicated in the restriction of a variety of pathogens through the induction of cell death pathways, the activation of an antimicrobial response within infected cells (RNS, ROS, antimicrobial peptides, etc.), the induction of autophagy, alterations in iron acquisition, and the induction of guanylate binding proteins (Bekker et al., 2001; Johnson and Wessling-Resnick, 2012; Pasparakis and Vandenabeele, 2015; Yamamoto et al., 2012; Yuk et al., 2012).

We narrowed our studies towards examining three possible mechanisms: apoptotic and necroptotic cell death, production of RNS and ROS, and induction of guanylate binding proteins. Autophagy is a possible mechanism of control; however, there is strong evidence that *C. burnetii* actively engages the autophagy pathway to promote its intracellular growth. Whether there is a role for host-driven modulation of iron levels in restricting *C. burnetii* infection is unclear, although a study suggested that *C. burnetii* actually replicated better within macrophage lines treated with an iron chelator compared to iron-replete macrophages (Briggs et al., 2008). It is possible that TNF-mediated alterations in phagocytic iron levels could still play a role in limiting *C. burnetii* replication.

We found *C. burnetii* does not induce cell death at measurable levels in C57BL/6 macrophages using both population-based and single cell-based assays, suggesting

that apoptosis and necroptosis are likely not major TNF-mediated control mechanisms *in vitro*. One possible caveat is that we did not use a single cell assay to detect necroptosis, so it is still possible that this form of cell death is occurring, and it is possible that necroptosis could mediate restriction of *C. burnetii* by occurring at an earlier time point in a small number of infected cells. However, since we observed the formation of large *C. burnetii*-containing vacuoles in a relatively large percentage of cells (~10-20%) in infected permissive macrophages lacking TLR2 or TNF, that would suggest that death of a small number of infected cells would not be sufficient to limit *C. burnetii* infection in our model. Since *C. burnetii* has been shown to inhibit intrinsic apoptosis through its T4SS, it is also possible that it uses bacterial effectors to inhibit extrinsic apoptosis and necroptosis. Studies where these pathways are artificially induced in *C. burnetii*-infected cells could aid in determining whether *C. burnetii* actively inhibits these cell death pathways and shed light on how *C. burnetii* is able to grow inside macrophages while inducing minimal cell death.

We did find that there was induction of both RNS and ROS in response to *C. burnetii* infection, and we further found RNS induction to be dependent on TNF. Future studies will determine whether ROS production is TNF-dependent. Our preliminary data also indicate that the NADPH oxidase, a major generator of ROS, plays a role in the restriction of *C. burnetii* replication in B6 macrophages, and future studies will determine whether RNS play a role in restricting *C. burnetii* replication in B6 macrophages. Studies with *S. Typhimurium*, which has a relatively short replication time within the host cell, showed ROS to be a major mediator of bacterial growth restriction (Mantena et al., 2008), while studies with *M. tuberculosis*, which has a longer replication time within the host cell, showed RNS to be a major restriction factor (Davis et al., 2007). Since *C.*

burnetii has a long replication time, it would be interesting to know if RNS plays a role in restriction at a later time point than ROS. These two pathways could target *C. burnetii* replication at different stages of its prolonged life cycle inside the cell.

We found that treatment of macrophages with TNF alone or *C. burnetii* infection of macrophages is capable of inducing two GBPs, GBP-1 and GBP-2, but did not find any evidence of a role for these or other GBPs in mediating growth restriction, as we did not observe increased *C. burnetii* replication when five of the eleven murine GBPs (GBP-1, -2, -3, -5, and -7) were absent. Further studies are necessary to implicate or definitively rule out GBPs as a potential effector mechanism underlying TNF-mediated bacterial restriction. Furthermore, since the *C. burnetii* vacuole can expand to occupy the majority of the cell cytosol and recruits membranes from a variety of sources, it would be interesting to know if any of the GBPs localize to the *C. burnetii*-containing vacuole.

Future Directions

Innate immune detection of Coxiella burnetii by cell surface and cytosolic sensors

Our findings open doors for many future studies aimed at examining the innate immune response to *C. burnetii* and its ability to evade innate immune detection. In continuing our first aim, short-term future directions should: define the exact signaling cascades activated by MyD88 and Trif signaling, determine whether TLR4 (or another TLR) is responsible for Trif-mediated signaling, and determine whether there is T4SS-mediated inhibition of these pathways. Long-term future directions should include: analysis of the activation of these innate immune pathways in human cell lines and primary human cells and *in vivo* studies in mice.

Further defining which specific downstream signaling pathways are activated (NF- κ B, MAPKs, IRFs) would offer a more complete picture of how TLRs and adaptors induce signaling cascades that lead to the expression of pro-inflammatory cytokines and other immune proteins. Since pro-inflammatory cytokine production can occur downstream of both NF- κ B and MAPK activation, a complete picture of *C. burnetii*'s activation of these pathways would be useful in determining its ability to manipulate or evade some of these innate immune responses.

We have not yet directly demonstrated that TLR4 mediates Trif signaling during *C. burnetii* infection. Furthermore, we found that purified *C. burnetii* LPS does not stimulate cytokine production in B6 macrophages, making it unclear how *C. burnetii* infection is triggering TLR4 signaling. Studies in cell lines ectopically expressing TLR4 or both TLR2 and TLR4 could shed light on determining whether TLR4 detects and signals through Trif in the context of the whole bacterium and whether TLR4 is sufficient to mediate responses to *C. burnetii*. It is also possible that TLR4-mediated detection of *C. burnetii* infection may somehow require an initial signal emanating from TLR2 detection. Thus, these studies should also include cell lines expressing both TLR2 and TLR4 to test this latter possibility.

Many intracellular bacteria utilize their secretion systems to translocate effector proteins that directly inhibit the activation of innate immune responses (Brodsky and Medzhitov, 2009). Since *C. burnetii* injects over 100 effector proteins, it is likely that some of these effectors target innate immune signaling pathways. Further studies could provide insight into whether there is T4SS-mediated inhibition of immune signaling. Comparing immune responses following artificial induction of immune pathways in infected cells versus uninfected cells would be one way to determine whether the T4SS

mediates immune inhibition, analogous to past studies showing that artificially induced intrinsic apoptosis is inhibited in a T4SS-dependent manner. For example, studies comparing responses to WT vs *icmL::Tn C. burnetii* NMII strains following induction of inflammasome activation with exogenous addition of ATP or induction of type I interferon with poly(I:C) would be useful. If cells infected with WT *C. burnetii* display less innate immune activation, then this would suggest that the T4SS translocates effectors that dampen immune signaling.

In the long-term, studies should examine innate immune responses to *C. burnetii* infection in human and murine macrophages. Humans and mice have evolved many divergent innate immune pathways. While our studies in mouse macrophages show the inflammasome is not activated and type I interferons are not induced during *C. burnetii* infection, it is possible that human immune sensors are able to respond to *C. burnetii* infection. One study with primary human alveolar macrophages showed that *C. burnetii* infection induces the release of IL-1 β , but did not implicate inflammasome activation in this system. Further studies in THP-1 cells and human primary macrophages would provide important insight into innate immune detection and control of *C. burnetii*.

Our studies only examined activation of innate immune pathways in macrophages. It is possible that different innate immune pathways are activated in other cells that would be expected to come into contact with *C. burnetii* in the lungs of an infected host, such as neutrophils, dendritic cells, and airway epithelial cells. Future studies examining the innate immune responses of these different cell types to *C. burnetii* would provide important insight into this question. Furthermore, future studies in mice using an intranasal model of infection could determine if these pathways are activated in systemic infection.

TNF as an Inhibitor of Coxiella burnetii Intracellular Replication

How TNF signaling restricts the intracellular replication of *C. burnetii* within host cells is unclear. To provide additional insight into the underlying mechanism, short-term future directions, building upon our initial findings, should: further determine whether RNS and ROS are responsible for the ability of TNF to mediate restriction and further analyze whether the mGBPs play a role in restricting *C. burnetii* infection,. Long term future directions should include examining the role of TNF in controlling *C. burnetii* growth and dissemination during *in vivo* infection.

TNF signaling can lead to cell death in other systems, but our data suggested that cell death was not robustly triggered during *C. burnetii* infection of macrophages. Examining *C. burnetii* replication in WT B6 macrophages compared to macrophages deficient in RIP3, which is required for necroptosis, or both RIP3 and caspase-8, which eliminates both extrinsic apoptosis and necroptosis, would provide important insight into whether cell death mediates TNF-dependent restriction of *C. burnetii* replication. Our data indicate that iNOS and the production of RNS are induced during *C. burnetii* infection in a TNF-dependent manner. Future experiments using RNS inhibitors or iNOS-deficient macrophages will aid in determining whether iNOS and RNS are involved in restricting *C. burnetii* replication. While we found that whole cell ROS was induced in response to *C. burnetii* infection, and our preliminary data suggest that the NADPH oxidase plays a role in restriction of *C. burnetii*, studies of ROS production in *Tnf*^{-/-} macrophages are necessary to link ROS-mediated restriction to TNF. It is also possible that TNF signaling somehow inhibits efficient T4SS translocation as a means to restrict

C. burnetii replication. Experiments that measure the magnitude of injection in WT and TNF-deficient macrophages using the BlaM translocation assay could help answer this.

Future studies should also examine whether GBPs are possible mediators of *C. burnetii* restriction within B6 macrophages, as our preliminary data indicate that at least two GBPs, mGBP-1 and mGBP-2, are transcriptionally induced during *C. burnetii* infection. GBPs have been implicated in restricting the intracellular replication of a variety of vacuolar pathogens, and further analysis of their expression and localization patterns and performing a targeted siRNA screen of the mGBPs could offer insight into their role during *C. burnetii* infection. While they have classically been thought to play a role in restriction of pathogens, it is also possible that their induction and recruitment to the vacuole may benefit *C. burnetii* and aid in its replication. *C. burnetii* is thought to engage with the autophagy pathway to promote its intracellular replication so it would be of interest to know if GBPs also fulfill a similar role.

It would also be of interest to determine which cells in the infected population are producing TNF. *L. pneumophila* and *S. flexneri* employ effector proteins that inhibit the production of pro-inflammatory cytokines by targeting host translational elongation factors and p38 and ERK MAPK signaling, respectively (Reiterer et al., 2011; Rolando and Buchrieser, 2014). In the case of *L. pneumophila*, it was recently shown using single cell assays that bystander cells are the primary producers of TNF and other cytokines (Copenhaver et al., 2015). Our studies thus far have relied largely on population-based assays, but single cell-based assays examining whether infected or uninfected cells produce cytokines would provide insight. Using the CCF4-AM/BlaM system in conjunction with mCherry-expressing *C. burnetii* would help divide the cells into uninfected, infected but uninjected, and T4SS-injected cells. A significant reduction in

cytokine-positive cells in the T4SS-injected population compared to uninjected cells would suggest that *C. burnetii* T4SS activity actively blocks production of proinflammatory cytokines.

Our studies focused solely on cell-intrinsic pathways of TNF restriction within macrophages during *in vitro* infection with the Nine Mile II strain, which is attenuated for virulence. It would also be of interest to determine the role for TNF in control of systemic infection with virulent *C. burnetii* strains. Intranasal infection of WT and TNF-deficient mice and measurement of bacterial burdens would offer a broader view of TNF as a control mechanism during *in vivo* infection. Since granulomas are thought to be important in the restriction of *C. burnetii* and may be linked to the formation of chronic infection, assays for granuloma formation in WT and TNF-deficient infected animals would be of great interest.

Finally, it is important to determine whether *C. burnetii* T4SS effectors participate in innate immune subversion. *C. burnetii* encodes over 100 effector proteins, many of which contain eukaryotic protein domains and unique localization patterns (Carey et al., 2011). Our discovery of various immune pathways that are not robustly activated during *C. burnetii* infection, such as inflammasome activation or the induction of type I IFNs, would offer possible phenotypes amenable to screening for T4SS effectors that inhibit these innate immune responses using gain-of-function or loss-of-function genetic approaches. Furthermore, advances in the field have allowed for the generation of transposon mutant libraries of effector proteins and it is now possible to test the roles of these effectors in inhibiting innate immune pathways in a similar fashion to the types of studies that have been performed in more genetically malleable bacteria.

Concluding Remarks

Innate immune recognition is a crucial first step in detecting and preventing microbial infection. Eukaryotic organisms have evolved specialized immune sensors that recognize a broad array of pathogen-associated molecular patterns (PAMPs). Detection of pathogens via these sensors activates cell-intrinsic and -extrinsic immune pathways to clear invaders. Many pathogens have evolved strategies to evade detection from these sensors in order to overcome innate immune barriers to infection. These evasion techniques can involve alterations in their PAMPs to become less immunostimulatory or the production of virulence factors that actively block key components of innate immune pathways. Intracellular bacteria pose a unique set of challenges for host organisms. They are shielded from circulating immune cells and molecules and also have evolved mechanisms to hijack host cells in order to form their replicative niche and evade intracellular detection. Much remains to be understood about how the innate immune system detects and controls the intracellular replication of this unique class of pathogens.

Coxiella burnetii is an intracellular bacterium that lives and grows in the lysosome, the harshest environment found within a cell. This bacterium can replicate in cells unchecked for days and can establish acute and chronic infections in humans. Unlike other bacteria, *C. burnetii* does not seem to induce robust innate immune activation. In these studies, we sought to define the innate immune pathways that *C. burnetii* does and does not activate in mouse macrophages, and understand the roles of these pathways in restricting *C. burnetii* infection. To address these questions, we chose to use restrictive B6 macrophages, which control *C. burnetii* replication, as a model for understanding the host factors involved in successful restriction of *C. burnetii* replication. We found that *C.*

burnetii infection does not robustly activate cytosolic immune sensors, the inflammasome, or the Type I interferon response. Instead, we found induction of pro-inflammatory cytokines and intracellular restriction of *C. burnetii* replication to be largely dependent on TLR signaling. The lack of a robust cytosolic immune response is remarkable because *C. burnetii* is capable of translocating bacterial effectors into the host cell cytosol of B6 macrophages. How it is able to accomplish effector translocation into host cells, while evading the slew of cytosolic immune sensors present within these cells, remains unknown, but it is likely *C. burnetii* has many strategies to overcome innate immune detection, potentially through its T4SS effectors.

Our findings also implicated endogenously produced TNF as a major mediator of cell-intrinsic immunity against *C. burnetii* within B6 macrophages. TNF is a broad-acting cytokine with a wide variety of downstream immune responses. We sought to determine the mechanism of TNF-mediated cell-intrinsic control by testing the role of several putative effector pathways known to be TNF-regulated in other systems. Our findings indicate that cell death likely does not play a major role in TNF-mediated restriction. Furthermore, our preliminary data suggest that several of the murine guanylate binding proteins are not required for TNF-mediated restriction. In addition, we further found that *C. burnetii* induces ROS and RNS, and our preliminary data suggest that the NADPH oxidase plays a role in cell-intrinsic control. Future studies are necessary to determine whether these pathways mediate the TNF-dependent restriction of *C. burnetii* infection, but our model system using C57BL/6-background macrophages deficient in select immune genes will allow us to test the role of these pathways in controlling *C. burnetii* infection.

Together, our studies suggest that *C. burnetii* does not robustly activate many innate immune pathways, but also offer new insight into possible mechanisms by which host cells are able to overcome *C. burnetii*'s subversion and restrict intracellular replication.

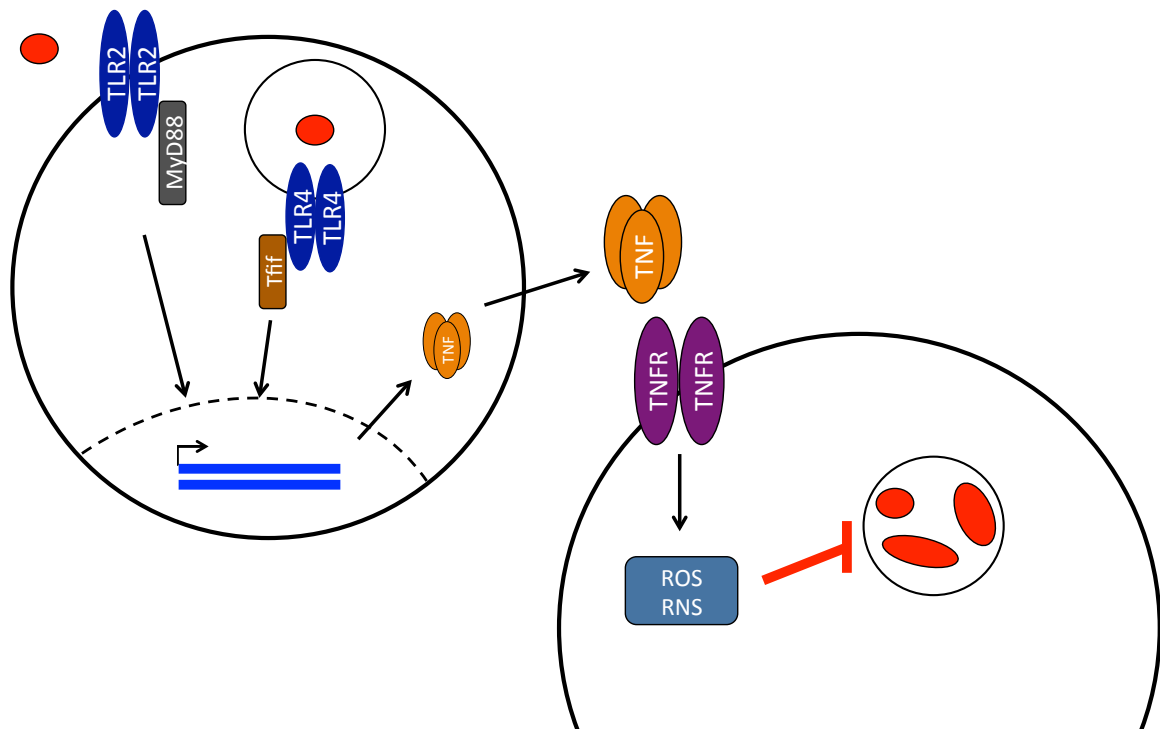


Figure 4.1. Proposed Mechanism of TLR-mediated control of *C. burnetii*

replication in C57BL/6 macrophages. Restriction of *C. burnetii* replication in C57BL/6 macrophages is dependent on TLR-mediated induction and secretion of TNF. TLR2 and TLR4 recognize *C. burnetii* and signal through both adaptors MyD88 and Trif to induce the production of TNF. TNF then binds to infected cells to induce the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which inhibit intracellular replication of *C. burnetii*. Restriction of replication in C56BL/6 is independent of cytosolic innate immune sensors.

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